1. Document ID: US 20010004757 A1

L1: Entry 1 of 24

File: PGPB

Jun 21, 2001

PGPUB-DOCUMENT-NUMBER: 20010004757
PGPUB-FILING-TYPE: new-utility
DOCUMENT-IDENTIFIER: US 20010004757 A1

TITLE: Processor and method of controlling the same

PUBLICATION-DATE: June 21, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Miyake, Hideo	Kawasaki		JP	
Suga, Atsuhiro	Kawasaki		JP	
Nakamura, Yasuki	Kawasaki		JP	

US-CL-CURRENT: 712/218; 712/226

AB: The present invention relates to a processor that performs a load operation prior to a store operation while avoiding ambiguous memory reference, and achieves high-speed operations. The present invention also relates to a method of controlling such a processor.

This processor includes a history control unit that stores a storage destination of a result obtained by executing a second instruction that is executed prior to a first instruction placed before the second instruction. When it is determined that the address of first data to be processed by the first instruction is included in the address region of second data to be processed by the second instruction, the history control unit overwrites the result obtained by the execution of the first instruction on the second data corresponding to the address.

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DOCUMENT-IDENTIFIER: US 20010004757 A1
TITLE: Processor and method of controlling the same

BSTX:
[0009] Meanwhile, the register control unit 7 includes an EPCR register 31, an EPSR register 33, a PSR register 35, the general register 37, and the floating point register 39. The EPCR register 31, the EPSR register 33, and the PSR register 35 are connected to an interrupt control circuit 40. The general register 37 is connected to the load instruction execution unit 19, the store instruction execution unit 21, and the instruction execution circuit 23. The floating point register 39 is connected to the floating point load instruction execution unit 25, the floating point store instruction execution unit 27, and the floating point arithmetic operation instruction execution unit 29.

BSTX:
[0018] When the instruction execution circuit 23 receives an arithmetic operation instruction from the instruction decoder unit 17, the instruction execution circuit 23 performs an operation based on a value read out from the general register 37, and writes the result in the general register 37. In a case where the instruction execution circuit 23 receives a branch instruction from the instruction decoder unit 17, the instruction execution circuit 23 supplies the branch destination address to the program counter 13 at the time of the occurrence of the branch. In a case where the instruction execution circuit 23 receives an interrupt return instruction, the instruction execution circuit 23 writes data that represents the pre-interrupt operation state in the register PSR 35. The instruction execution circuit 23 then reads the address of the instruction at the return destination from the register EPCR 31, and supplies the address as the branch destination address to the program counter 13. However, if the instruction execution circuit 23 detects an interrupt while executing the above instruction, the instruction execution circuit 23 supplies an interrupt signal to the interrupt control circuit 40.

BSTX:
[0019] The register EPCR 31 holds the address of an instruction corresponding to the return destination from the interrupt. The address is set at the occurrence of the interrupt. The register PSR 35 holds data that represents the operation state, and the register EPSR 33 holds data that represents the pre-interrupt operation state set prior to the occurrence of the interrupt.

BSTX:
[0020] Based on the interrupt signal supplied from the instruction read unit 3 or the instruction execution unit 5, the interrupt control circuit 40 writes the instruction address corresponding to the interrupt return destination in the register EPCR 31, the data that represents the pre-interrupt operation state in the register EPSR 33, and the operation state corresponding to the interrupt in the PSR 35. The interrupt control circuit 40 supplies the branch destination address corresponding to the interrupt to the instruction read unit 3.

BSTX:
[0022] When an interrupt occurs, the interrupt control circuit 40 writes the instruction address corresponding to the interrupt return destination in the register EPCR 31, the data that represents the pre-interrupt operation state in the EPSR 33, and the operation state of the interrupt in the PSR 35, based on the interrupt signal supplied from the instruction read unit 3 or the instruction execution unit 5. Also, the interrupt control circuit 40 supplies the branch destination address corresponding to the interrupt to the instruction read unit 3. The instruction read unit 3 then reads out an instruction word in accordance with the branch destination address supplied from the interrupt control unit 9, and supplies the instruction word to the instruction execution unit 5.
After that, the operation is carried out in the same manner as in the normal state described above.

BSTX:
[0023] At the time of interrupt return, the instruction execution unit 5 executes an interrupt return instruction, thereby writing the value of the register EPSR 33 in the register PSR 35. The instruction execution unit 5 reads out the data from the register EPCR 31,

and supplies the result
as the branch destination address to the instruction read unit 3. The instruction read unit 3 in
turn reads out an instruction word in accordance with the branch destination address supplied from
the instruction execution unit 5, and supplies the instruction word to the instruction execution
unit 5. After that, the operation is performed in the same manner as in the above-described normal
state.

2. Document ID: US 6294368 B1

L1: Entry 2 of 24 File: USPT Sep 25, 2001

US-PAT-NO: 6294368
DOCUMENT-IDENTIFIER: US 6294368 B1

TITLE: Isolated human metalloprotease proteins, nucleic acid molecules encoding human protease
proteins, and uses thereof

DATE-ISSUED: September 25, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Merkulov; Gennady V.	Baltimore	MD	N/A	N/A
Ye; Jane	Boyds	MD	N/A	N/A
Di Francesco; Valentina	Rockville	MD	N/A	N/A
Beasley; Ellen M.	Darnestown	MD	N/A	N/A

US-CL-CURRENT: 435/219; 435/320.1, 435/69.1, 536/23.2, 536/23.5

AB: The present invention provides amino acid sequences of peptides that are encoded by
genes within the human genome, the protease peptides of the present invention. The present
invention specifically provides isolated peptide and nucleic acid molecules, methods of
identifying orthologs and paralogs of the protease peptides, and methods of identifying
modulators of the protease peptides.

L1: Entry 2 of 24 File: USPT Sep 25, 2001

DOCUMENT-IDENTIFIER: US 6294368 B1
TITLE: Isolated human metalloprotease proteins, nucleic acid molecules encoding human protease
proteins, and uses thereof

DEPR:
Full-length pre-processed forms, as well as mature processed forms, of proteins that comprise one of
the peptides of the present invention can readily be identified as having complete sequence identity
to one of the protease peptides of the present invention as well as being encoded by the same
genetic locus as the protease peptide provided herein. As indicated by the data presented in FIG. 3,
the map position was determined to be on chromosome 1 by ePCR.

DEPR:
Allelic variants of a protease peptide can readily be identified as being a human protein having a
high degree (significant) of sequence homology/identity to at least a portion of the protease
peptide as well as being encoded by the same genetic locus as the protease peptide provided herein.
Genetic locus can readily be determined based on the genomic information provided in FIG. 3, such as
the genomic sequence mapped to the reference human. As indicated by the data presented in FIG. 3,
the map position was determined to be on chromosome 1 by ePCR. As used herein, two proteins (or a
region of the proteins) have significant homology when the amino acid sequences are typically at
least about 70-80%, 80-90%, and more typically at least about 90-95% or more homologous. A
significantly homologous amino acid sequence, according to the present invention, will be encoded by
a nucleic acid sequence that will hybridize to a protease peptide encoding nucleic acid molecule
under stringent conditions as more fully described below.

DEPR:
Orthologs of a protease peptide can readily be identified as having some degree of significant
sequence homology/identity to at least a portion of the protease peptide as well as being encoded by
a gene from another organism. Preferred orthologs will be isolated from mammals, preferably
primates, for the development of human therapeutic targets and agents. Such orthologs will be
encoded by a nucleic acid sequence that will hybridize to a protease peptide encoding nucleic acid
molecule under moderate to stringent conditions, as more fully described below, depending on the
degree of relatedness of the two organisms yielding the proteins. As indicated by the data presented
in FIG. 3, the map position was determined to be on chromosome 1 by ePCR.

DEPR:
The nucleic acid molecules are also useful as probes for determining the chromosomal positions of
the nucleic acid molecules by means of in situ hybridization methods. As indicated by the data
presented in FIG. 3, the map position was determined to be on chromosome 1 by ePCR.

DEPR:
Individuals carrying mutations in the protease gene can be detected at the nucleic acid level by a
variety of techniques. FIG. 3 provides information on SNPs that have been found in the gene encoding
the protease protein of the present invention. SNPs were identified at 7 different nucleotide
positions in introns and regions 5' and 3' of the ORF. Such SNPs in introns and outside the ORF may
affect control/regulatory elements. As indicated by the data presented in FIG. 3, the map position
was determined to be on chromosome 1 by ePCR. Genomic DNA can be

analyzed directly or can be
amplified by using PCR prior to analysis. RNA or cDNA can be used in the same way. In some uses,
detection of the mutation involves the use of a probe/primer in a polymerase chain reaction (PCR)
(see, e.g. U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or,
alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al., Science
241:1077-1080 (1988); and Nakazawa et al., PNAS 91:360-364 (1994)), the latter of which can be
particularly useful for detecting point mutations in the gene (see Abravaya et al., Nucleic Acids
Res. 23:675-682 (1995)). This method can include the steps of collecting a sample of cells from a
patient, isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample,
contacting the nucleic acid sample with one or more primers which specifically hybridize to a gene
under conditions such that hybridization and amplification of the gene (if present) occurs, and
detecting the presence or absence of an amplification product, or detecting the size of the
amplification product and comparing the length to a control sample. Deletions and insertions can be
detected by a change in size of the amplified product compared to the normal genotype. Point
mutations can be identified by hybridizing amplified DNA to normal RNA or antisense DNA sequences.

3. Document ID: US 6200751 B1

L1: Entry 3 of 24 File: USPT Mar 13, 2001

US-PAT-NO: 6200751
DOCUMENT-IDENTIFIER: US 6200751 B1

TITLE: Endothelial specific expression regulated by EPCR control elements

DATE-ISSUED: March 13, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gu; Jian-Ming	Oklahoma City	OK	N/A	N/A
Esmon; Charles T.	Oklahoma City	OK	N/A	N/A

US-CL-CURRENT: 435/6; 536/24.1

AB: The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and
human (SEQ. ID No. 2) genomic libraries. The promoter includes a region (nucleotides 3130 to
3350 of SEQ. ID No. 1 which affects selective gene expression in endothelial cells), and a
region (nucleotides 2270 to 2840 of SEQ. ID No. 1) which affects selective gene expression in
large vessel endothelial cells, as compared to expression in all endothelial cells. The EPCR
promoter contains a thrombin responsive element, CCCACCCC (SEQ. ID No. 3), (murine, nucleotides
3007 to 3014 SEQ. ID No. 1 and human, nucleotides 2722 to 2729 SEQ. ID No. 2). The EPCR also
contains a serum response element (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory
sequences present in the EPCR promoter can be used for thrombin or serum controlled recombinant
gene expression specific to either all endothelial cells or primarily endothelial cells of
large vessels. Therapeutic strategies include the use of the minimal promoter for expression of
therapeutic agents during times of increased thrombin/platelet activation or regional trauma in
all endothelial cells or the use of the large vessel specific promoter for regional specific
expression in the endothelial cells of large vessels for use in delivery.

L1: Entry 3 of 24 File: USPT Mar 13, 2001

DOCUMENT-IDENTIFIER: US 6200751 B1
TITLE: Endothelial specific expression regulated by EPCR control elements

ABPL:
The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID
No. 2) genomic libraries. The promoter includes a region (nucleotides 3130 to 3350 of SEQ. ID No. 1
which affects selective gene expression in endothelial cells), and a region (nucleotides 2270 to
2840 of SEQ. ID No. 1) which affects selective gene expression in large vessel endothelial cells, as
compared to expression in all endothelial cells. The EPCR promoter contains a thrombin responsive
element, CCCACCCC (SEQ. ID No. 3), (murine, nucleotides 3007 to 3014 SEQ. ID No. 1 and human,
nucleotides 2722 to 2729 SEQ. ID No. 2). The EPCR also contains a serum response element
(nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter
can be used for thrombin or serum controlled recombinant gene expression specific to either all
endothelial cells or primarily endothelial cells of large vessels. Therapeutic strategies include
the use of the minimal promoter for expression of therapeutic agents during times of increased
thrombin/platelet activation or regional trauma in all endothelial cells or the use of the large
vessel specific promoter for regional specific expression in the endothelial cells of large vessels
for use in delivery.

PCPR:
This application claims the benefit of U.S. Provisional application Ser. No. 60/030,718 filed Nov.
8, 1996, by Charles T. Esmon, Wei Ding, Jian-Ming Gu and Kenji Fukudome, entitled "Thrombin Response
Element", and U.S. Ser. No. 60/054,533 filed Aug. 4, 1997 by Charles T. Esmon and Jian-Ming Gu,
entitled "Endothelial Specific Expression Regulated by EPCR Control Elements".

BSPR:
The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID
No. 2) genomic libraries. The promoter has been demonstrated to include a region which results in
selective expression in endothelial cells, between -1 and -220 based on the positions relative to

the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ.
ID No. 1), and a region which selectively results in expression in large vessel endothelial cells,
as opposed to all endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of
SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR promoter, from -337 to
-345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368
(nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID
No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to
3061 of SEQ. ID No. 1).

BSPR:
The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding
other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin
or serum or to effect selective expression in endothelial cells generally or preferentially in
endothelial cells of the large blood vessels. The gene control elements include elements responsive
to environmental stimuli (either thrombin or serum); and information to determine distribution of
the desired protein expression (large vessels). Therapeutic strategies include the use of the
minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of
gene therapy where systemic distribution is desired; the use of a promoter including an
environmental stimuli response element(s), for use in delivery of agents whose expression should be
increased during times of increased thrombin/platelet activation or during regional trauma; the use
of the minimal promoter including an environmental stimuli response element and the element
directing expression to large vessel endothelium, where a response to regional trauma is desirable
but only in large vessel endothelium, and the use of the minimal promoter and element directing
expression to large vessel endothelium, where expression is specifically targeted to large vessel
endothelium but is not increased in response to any particular stimuli.

DRPR:
FIGS. 1A-1B are a comparison of the nucleotide sequences of the murine EPCR (nucleotides 2993 to
3481 of SEQ ID No. 1) and human EPCR promoters (nucleotides 2704 to 3224 of SEQ. ID No. 2).

DRPR:
FIG. 4 is a schematic of the promoter. The top line indicates the structure of the promoter from
-220 to -177, (showing nucleotides 3120 to 3157 of SEQ ID No. 1) which includes the transcription
control elements required for constitutive expression in endothelial cells (nucleotides 3120 to 3156
of SEQ ID No. 1). AP4 and SP-1 are known promoter elements that bind proteins that control gene
expression. The bottom line is a schematic representation of the EPCR promoter showing the locations
of the large vessel specific element between -1080 and -700 ("C"), the element which includes the
sequence responsible for thrombin induction ("B"), the endothelial specific region ("A"), and the
EPCR encoding element. SP-1, AP-1 and AP-4 are known promoter elements which bind proteins involved
in transcription control.

DEPR:
Specific targeting of expression of desired genes can be achieved through the selection and use of
the regulatory sequences described herein in detail, isolated from the protein C receptor (EPCR).
The protein C receptor is the first protein identified and reported with these properties. It is
expressed in high levels exclusively in large vessels, and the expression levels decrease with
vessel size, until there is little-to-no expression detectable in capillaries.

DEPR:
The EPCR Regulator Sequences

DEPR:
The endothelial cell protein C binding protein (referred to herein as "EPCR") was cloned and
characterized, as described in PCT/US95/09636 "Cloning and Regulation of an Endothelial Cell Protein
C/Activated Protein C Receptor" Oklahoma Medical Research Foundation. The protein was predicted to
consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a
23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane
protein. The protein binds with high affinity to both protein C and activated protein C (Kd=30 nM),
which is a naturally occurring anticoagulant, and is calcium dependent.

DEPR:
Following identification and cloning of the endothelial cell protein C receptor (EPCR), it was
determined that the EPCR was down regulated in cultured endothelial cells by TNF.alpha.. To
determine the physiological relevance of this finding, EPCR mRNA levels in rats and mice challenged
with LD.sub.95 levels of endotoxin were examined. Surprisingly, in response to endotoxin infusion,
EPCR message rose within three hours to about four fold the basal level and remained elevated for
twelve hours, then returning toward baseline at 24 hours. The rapid response suggested that a factor
generated by endotoxin infusion could upregulate EPCR expression. Since thrombin is known to be one
of these factors, rat microvascular cells in culture were treated with thrombin (0.1 units/ml). The
cells exhibited a three to four fold increase in EPCR mRNA levels within three hours relative to
control cells.

DEPR:
Physiologically, these results showing elevated mRNA levels three hours after exposure to thrombin,
which begins to decline after twelve hours to baseline levels by 24 hours, are important since they
suggest that thrombin plays a direct in vivo role in upregulation of EPCR expression. High level
EPCR expression could contribute to the decrease observed in protein C levels during acute
inflammatory response syndromes.

DEPR:
The gene encoding EPCR including the promoter region was then isolated from a murine genomic
library, using the DNA encoding murine EPCR as a probe. A human genomic library was similarly
screened with the DNA encoding human EPCR to isolate the promoter for the human EPCR Analysis of the
promoter revealed a thrombin response element. Gel shift assays revealed that thrombin treatment
induced at least one factor that binds specifically to this promoter region. Further analysis
yielded the sequence of the thrombin responsive element. This element can be used to increase
selective expression in response to thrombin. The promoter is also selective in expression, with the
EPCR being selectively expressed more in large vessel endothelial cells when most of the entire
promoter is present, including the beginning region. When a shorter portion of the promoter is
present, there is expression in all endothelial cells. These results are consistent with a repressor
being present in the first part of the promoter which suppresses expression in capillary endothelial

cells.

DEPR:
Referring to FIGS. 1A-1B and SEQ. ID Nos 1 (the murine EPCR promoter) and 2 (the human EPCR promoter), the 5' regulatory sequences of the EPCR includes a transcription initiation promoter specific to endothelium contained in -1 to -220 (nucleotides 3130 to 3350 of SEQ. ID No. 1) (referred to for ease of reference as "A"), a control element responsive to thrombin (CCCACCCC) (SEQ. ID No. 3) located between -337 and -345 in the murine promoter (nucleotides 3007 to 3014 of SEQ ID No. 1) and between -360 and -368 in the human promoter (nucleotides 2722 to 2729 of SEQ. ID No. 2) (referred to as "B"), a serum response element located between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1) (referred to as "D"), and a large vessel expression element located between -1080 and -700 (nucleotides 2270 to 2840 of SEQ. ID No. 1) (referred to as "C"). The latter directs expression primarily to large vessels such as aorta, coronary arteries, arteries and veins, rather than to capillaries.

DEPR:
FIGS. 1A-1B are a comparison of the sequences from the murine and human promoters, demonstrating that they are highly homologous. It is understood that the equivalent regions from the promoters of EPCR from other species could be used to achieve the same type of expression, and that sequences from different species could be used in combination, for example, A from the murine promoter and C from the human promoter.

DEPR:
The nucleotide sequences are important as hybridization probes, in selected expression of recombinant proteins other than EPCR, in increasing expression of recombinant proteins by exposure of the encoding construct to thrombin, and in design and screening of drugs and diagnostics for therapeutic and research purposes.

DEPR:
The constructs are particularly useful in gene therapy. The elements can be used to regulate expression of a gene encoding an important protein, or a biologically active nucleic acid molecule such as antisense, triplex forming molecules, ribozymes, and guide sequences for RNAase P which can be used to mutate or stop transcription of a particular gene. Examples of gene targeting include expression of thrombomodulin (TM), EPCR, TFPI, tPA, or heparin (heparan proteoglycans) in large vessel endothelium to decrease clot propensity at atheromas or in autoimmune diseases. If systemic elevations of tPA was desired, sequence A could be used on the gene. Endogenous gene expression could be suppressed by using sequence A, ABC or possibly AC, coupled to antisense to block expression of adhesion molecules to decrease leukocyte infiltration in atherosclerosis. The thrombin response element is significantly inducible in vivo, and should therefore be particularly useful in the treatment of patients with a history of constitutively elevated levels of thrombin, for example, particularly for expression of therapeutic genes in coronary arteries in patients with unstable angina.

DEPR:
Nucleotide sequences were determined for 8.8 kb of the genomic structure and 3.4 kb of the 5'-flanking region of the mouse EPCR (mEPCR) gene. RNase protection assay revealed six major transcription start sites clustered at -110 to -119 upstream of the translation initiation site. A series of 5'-promoter deletion fragments: mP3340, mP1120, mP700, mP350 and an SV40 control were fused to a luciferase reporter gene and transiently transfected into several cell types, bovine aorta endothelial cells (large vessel endothelial cells), rat heart endothelial cells which is mostly capillary endothelial cells (small vessel endothelial cells), and 293 kidney cells (non-endothelial cells).

DEPR:
Transgenic mice were developed using either the -350 to -1 or -1080 to -1 regions of the mouse EPCR promoter to drive the structural gene for green fluorescent protein (GFP) to determine the in vivo activity of the previously described promoter regions.

DEPR:
The promoter regions (-1080 and -350) of mouse EPCR gene were cloned into the pEGFP1 vector (Clontech), which already contains the structural gene for GFP. The fragments which contained the promoter region of mEPCR and GFP reporter gene were released by enzymes Eco47 III and Afl II from the constructs pEGFP350 and pEGFP1080. After purification, the DNA fragments were microinjected into the pronuclei of fertilized mouse eggs by standard methods. Mice were screened for the presence of the transgene by GFP specific PCR and Southern blotting by standard methods. Several transgenic lines were established from both promoter constructs.

DEPR:
GFP mRNA was constitutively expressed in these lines. The level of GFP mRNA expression was variable from significantly less than to higher than the endogenous EPCR expression. These data indicate that the ability to express a foreign structural gene under the control of these promoters will not be chromosome integration position dependent, although constitutive level of expression may be influenced by chromosomal positioning.

DEPC:
Example 3: In vivo Activity of the EPCR Promoter.

CLPR:
1. An isolated naturally occurring cell specific regulatory element present in the endothelial protein C receptor promoter which directs expression to human, bovine, rat or murine endothelial cells, wherein said regulatory element is contained in nucleotides 3130 to 3350 of SEQ ID NO:1 or is contained in nucleotides 2868 to 3087 of SEQ ID NO:2.

CLPR:
4. An isolated naturally occurring tissue specific regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to human, bovine, rat or murine large vessel endothelial cells, wherein said regulatory element is contained in nucleotides 2270 to 2840 of SEQ ID NO:1 or is contained in nucleotides 2008 to 2388 of SEQ ID NO:2.

CLPR:
7. An isolated inducible regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum wherein said regulatory element is contained in nucleotides 2990 to 3061 of SEQ ID NO:1 or is contained in nucleotides 2738 to 2808 of SEQ ID NO:2.

CLPR:
10. A construct for expression of a gene comprising a regulatory element selected from the group consisting of an isolated naturally occurring regulatory element present in

the endothelial protein
C receptor promoter which directs expression to human, bovine, rat or murine endothelial cells,
which is contained in nucleotides 3130 to 3350 of SEQ ID NO:1 an isolated naturally occurring
regulatory element present in the endothelial protein C receptor promoter which preferentially
directs expression to human, bovine, rat or murine large vessel endothelial cells, which is
contained in nucleotides 2270 to 2840 of SEQ ID NO:1, or which is contained in nucleotides 2008 to
2388 of SEQ ID NO:2, and an isolated regulatory element present in the endothelial protein C
receptor promoter which is inducible by exposure to serum, which is contained in nucleotides 2990 to
3061 of SEQ ID NO:1 or is contained in nucleotides 2738 to 2808 of SEQ ID NO:2.

CLPR:
13. The construct of claim 10 wherein the regulatory element comprises an isolated regulatory
element present in the endothelial protein C receptor promoter which directs expression to human,
bovine, rat or murine endothelial cells, which is contained in nucleotides 3130 to 3350 of SEQ ID
NO:1, or which is contained in nucleotides 2868 to 3087 of SEQ ID NO:2, and an isolated regulatory
element present in the endothelial protein C receptor promoter which preferentially directs
expression to human, bovine, rat or murine large vessel endothelial cells, which is contained in
nucleotides 2270 to 2840 of SEQ ID NO:1 or which is contained in nucleotides 2008 to 2388 of SEQ ID
NO:2.

CLPR:
15. A method for controlling the expression of a gene comprising expressing the gene under the
control of a regulatory element selected from the group consisting of an isolated naturally
occurring regulatory element present in the endothelial protein C receptor promoter which directs
expression to human, bovine, rat or murine endothelial cells, which is contained in nucleotides 3130
to 3350 of SEQ ID NO:1, or which is contained in nucleotides 2868 to 3087 of SEQ ID NO:2, an
isolated naturally occurring regulatory element present in the endothelial protein C receptor
promoter which preferentially directs expression to human, bovine, rat or murine large vessel
endothelial cells, which is contained in nucleotides 2270 to 2840 of SEQ ID NO:1, or which is
contained in nucleotides 2008 to 2388 of SEQ ID NO:2, and an isolated regulatory element present in
the endothelial protein C receptor promoter which is inducible by exposure to serum, which is is
contained in nucleotides 2990 to 3061 of SEQ ID NO:1 or which is contained in nucleotides 2738 to
2808 of SEQ ID NO:2.

CLPR:
17. The method of claim 15 wherein the gene is expressed under the control of an isolated naturally
occurring regulatory element present in the endothelial protein C receptor promoter which directs
expression to human, bovine, rat or murine endothelial cells, which is contained in nucleotides 3130
to 3350 of SEQ ID NO:1, or which is contained in nucleotides 2868 to 3087 of SEQ ID NO:2, and an
isolated regulatory element present in the endothelial protein C receptor promoter which
preferentially directs expression to human, bovine, rat or murine large vessel endothelial cells,
which is contained in nucleotides 2270 to 2840 of SEQ ID NO:1, or which is contained in nucleotides
2008 to 2388 of SEQ ID NO:2.

ORPL:
Fukudome, et al., "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein
C/Activated Protein C Receptor (EPCR)," J. Biological Chemistry 270(10):5571-5577 (1995).

ORPL:
Gu, et al., "Functional Characterization of 5'-Flanking Region of the Mouse Endothelial Protein C
Receptor (EPCR) Gene," Mol. Biol. Cell Supp. 8:228a, Abstract No. 1323 (1997).

4. Document ID: US 6180355 B1

L1: Entry 4 of 24 File: USPT Jan 30, 2001

US-PAT-NO: 6180355
DOCUMENT-IDENTIFIER: US 6180355 B1

TITLE: Method for diagnosing and treating chronic pelvic pain syndrome

DATE-ISSUED: January 30, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Alexander; Richard B.	Ellicott City	MD	N/A	N/A
Ponniah; Sathibalan	Ellicott City	MD	N/A	N/A

US-CL-CURRENT: 435/7.1; 435/7.8

AB: The present invention provides a superior method of diagnosing Chronic Pelvic Pain
Syndrome in men comprising measuring levels of cytokines in semen or components or fractions of
semen. The invention also provides a method of treating a condition associated with elevated
levels of a cytokine, such as TNF-.alpha., in semen or a component or fraction thereof,
comprising administering a therapeutically effective amount of an ant-cytokine compound or
composition, such as an anti-TNF-.alpha. compound or composition.

L1: Entry 4 of 24 File: USPT Jan 30, 2001

DOCUMENT-IDENTIFIER: US 6180355 B1
TITLE: Method for diagnosing and treating chronic pelvic pain syndrome

DETL:
formation of prohormone fragment(s) by proteolysis of the mediated disease prohormone, and uses of
the antibody including prophylactic and therapeutic methods to treat disease, and diagnostic assays

for determining the amount of the prohormone and prohormone fragments present in a patients body.

5,700,909 Dec 23, Prosaposin and cytokine- Prosaposin and peptide derivatives derived therefrom will promote neurite outgrowth in 1997 derived peptides vitro. A peptide consensus sequence was determined by comparing the active neurite outgrowth-inducing saposin C peptide sequence with that of various hematopoietic and neuropoietic cytokines. These cytokine-derived peptides will promote the same processes as their corresponding cytokines. In addition, prosaposin and saposin C promote increased nerve cell myelination ex vivo. 5,700,788 Dec. 23, Ureido derivatives of Subject of the present invention are new ureido derivatives of naphthalenephosphonic 1997 naphthalenephosphonic acids having the following formula (I) [See Original Patent for Chemical Structure Diagram] acids (I) [See Original Patent for Chemical Structure Diagram] wherein each of m and n, which are the same, is an integer of 1 to 4; each of p and q, which are the same, is an integer of 1 gto 3; and each of the R groups, which are the same, is a free or esterified phosphonic acid group; and the pharmaceutically acceptable salts thereof. 5,698,711 Dec. 16, Compounds containing This invention is directed to the pharmaceutical use of phenyl compounds, which are 1997 phenyl linked to aryl or linked to an aryl moiety by various linkages, for inhibiting tumor necrosis factor. The heteroaryl by an aliphatic- invention is also directed to the compounds, their preparation and pharmaceutical or heteroatom-containing compositions containing these compounds. Furthermore, this invention is directed to the linking group pharmaceutical use of the compounds for inhibiting cyclic AMP phosphodiesterase. 5,698,706 Dec. 16, Heterocyclic amides and Peptidyl derivatives having a SH or acyl S group and which are amides, primary amides or 1997 methods of use thioamides, have therapeutic utility via MMP or TNF inhibition. 5,698,579 Dec. 16, Cyclic amides Cyclic amides are inhibitors of tumor necrosis factor and can be used to combat cachexia, 1997 endotoxic shock, and retrovirus replication. A typical embodiment is 3-phenyl-3-(1-oxoisoindolin-2-yl)propionamide.

5,698,564 Dec. 16, Diphenyl disulfide Diphenyl disulfide compounds having an inhibiting activity against the production of 1997 compounds Interleukin-1 beta (IL-1 beta) or the release of Tumor Necrosis Factor alpha (TNF alpha), which are useful in the treatment or prophylaxis of the diseases such as chronic rheumatism and sepsis are described. 5,698,518 Dec. 16, Method for regulating A method of treating patients to inhibit inflammation is disclosed. In the method, an 1997 inflammation and tumor effective amount of calmodulin, a calmodulin analogue or calmodulin receptor agonist is growth with calmodulin, administered to a patient to inhibit production of tumor necrosis factor and/or augment calmodulin analogues or elastase. In another method, an effective amount of calmodulin antagonist is administered calmodulin antagonists to a patient to stimulate immune response or inhibit elastase release. In another embodiment, a diagnostic test is disclosed to be used on patient blood samples to determine individual propensity to regulate tumor necrosis factor and/or elastase by calmodulin, its analogues or receptor agonists. 5,698,391 Dec 16, Methods for synthetic Methods useful for the determination of oligomers which have specific activity for a target 1997 unrandomization of molecule from a pool of primarily randomly assembled oligomers are provided. The oligomer fragments disclosed methods involve repeated syntheses of increasingly simplified sets of oligomers coupled with selection procedures for determining oligomers having the highest activity. Freedom from the use of enzymes allows the application of these methods to any molecules which can be oligomerized in a controlled fashion. 5,698,195 Dec. 16, Methods of treating Anti-TNF antibodies, fragments and regions thereof which are specific for human tumor 1997 rheumatoid arthritis using necrosis factor- alpha (TNF alpha) and are useful in vivo for diagnosis and therapy of a chimeric anti-TNF number of TNF alpha -mediated pathologies and conditions, including rheumatoid arthritis antibodies as well as polynucleotides coding for murine and chimeric antibodies, methods of producing the antibody, methods of use of the anti-TNF antibody, or fragment, region or derivative thereof, in immunoassays and immunotherapeutic approaches are provided.

5,695,993 Dec. 9, Cloning and regulation of Human protein C and activated protein C were shown to bind to endothelium specifically, 1997 an endothelial cell protein selectively and saturably (Kd = 30 nM, 7000 sites per cell) in a Ca<2 +> dependent C/activated protein C fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type receptor transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF.

Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response. 5,695,953 Dec. 9, DNA that encodes a tumor Tumor Necrosis Factor (TNF) Inhibitory Protein is isolated and substantially purified and 1997 necrosis factor inhibitory the DNA that encodes the TNF inhibitory protein, vectors, host cells, and a recombinant protein and a recombinant method for producing the encoded protein are also set forth.

It has the ability to inhibit: (a) method of production the binding of TNF to its receptors, and (b) the cytotoxic effect of TNF. TNF Inhibitory Protein and salts, functional derivatives and active fractions thereof can be used to antagonize the deleterious effects of TNF. 5,691,382 Nov. 25, Inhibition of TNF production The present invention is directed to the method of inhibiting the release of tumor necrosis 1997 with matrix factor (TNF) in a condition mediated by TNF by administration of certain hydroxamic add metaloproteinase inhibitors derivatives, also known as matrix metalloproteinase inhibitors, and thus the method of this invention is useful in the management of diseases or conditions mediated by TNF. 5,691,381 Nov. 25, Hydroxamic and The present invention provides novel hydroxamic acids and carbocyclic acids and 1997 carbocyclic acids as derivatives thereof and to pharmaceutical compositions and methods of use of these novel metalloprotease inhibitors compounds for the inhibition of matrix metalloproteinases, such as stromelysin, and inhibit the production of tumor necrosis factor alpha, and for the treatment of arthritis and other related inflammatory diseases. these novel compounds are represented by Formula I below [See Original Patent for Chemical Structure Diagram] Formula I 5,688,805 Nov. 18, Tricyclic derivatives, Disclosed are compounds of Formula [See Original Patent for Chemical Structure 1997 compositions and methods Diagram] (I) or a pharmaceutically acceptable salt or solvate thereof. Also disclosed are of use pharmaceutical compositions containing compounds of Formula I, methods for inhibiting tumor necrosis factor- alpha and methods for treating septic shock, inflammation, or

allergic disease. 5,686,455 Nov. 11, Imidazole derivatives and As cytokine inhibitors
2,4,5-triarylimidazole compounds and compositions for use as 1997 their use as cytokine cytokine
inhibitors. inhibitors 5,686,431 Nov. 11, Methods of using low The present invention relates to
methods for the prevention and/or treatment of 1997 molecular weight heparins pathological processes
involving the induction of TNF- alpha secretion comprising a for treatment of pathological
pharmaceutically acceptable carrier and a low molecular Weight heparin (LMWH). In the processes
pharmaceutical compositions of the present invention, the LMWH present in a low effective dose and
is administered at intervals of about 5-8 days. Furthermore, the LMWH is capable of inhibiting in
vitro TNF- alpha secretion by resting T cells and/or macrophages in response to T cell-specific
antigens, mitogens, macrophage activators, disrupted extracellular matrix (dECM), laminin,
fibronectin, and the like. 5,686,259 Nov. 11, Assay method for the Cleavage site blocking antibody
that binds to prohormones, preferable Tumor Necrosis 1997 detection of 26 kd TNF Factor, thereby
preventing the formation of prohormone fragment(s) by proteolysis of the prohormone prohormone, and
uses of the antibody including prophylactic and therapeutic methods to treat disease, and diagnostic
assays for determining the amount of the prohormone and prohormone fragments present in a patients
body. 5,684,222 Nov. 4, Mutant mouse having a The multiple biological activities of tumor necrosis
factor (TNF) are mediated by two 1997 disrupted TNFRp55 distinct cell surface receptors of 55 and 75
kDa. Mutant mice of the invention lacking tumor necrosis factor receptor (TNFR) p55 still express
functional TNFRp75 molecules at the cell surface. Normal weight and size of the mutant mice are not
altered. Thymocyle development and lymphocyle populations are normal, and clonal deletion of
potentially self-reactive T cells is not impaired. Activation of the nuclear transcription factor
kappa B (NF- kappa B), however, is completely abrogated after stimulation with TNF. Moreover,
TNFRp55 mutant mice are protected from septic shock induced by bacterial endotoxin or superantigen,
but Listeria clearance is severely impaired and mutant mice easily succumb to Listeria infection.
Thus, the two TNF receptors are not redundant, are independently controlled, and piay different
roles in normal and pathological physiology. 5,679,696 Oct 21, Compounds containing This invention
is directed to the pharmaceutical use of phenyl compounds, which are 1997 phenyl linked to aryl or
linked to an aryl moiety by vanous linkages, for inhibiting tumor necrosis factor. The heteroaryl by
an aliphatic-or invention is also directed to the compounds, their preparation and pharmaceutical
heteroatom-containing compositions containing these compounds. Furthermore, this invention is
directed to the linking group pharmaceutical use of the compounds for inhibiting cyclic AMP
phosphodiesterase. 5,679,684 Oct. 21, Hydroxyalkylammonium- Novel hydroxyalkylammonium-pyrimidine of
the formula [See Original Patent for Chemical 1997 pyrimidines and nucleoside Structure Diagram] (I)
and nucieoside derivatives have been found to be useful as derivatives, useful as inhibitors of
inflammatory cytokines. They can be used, inter alia, in the therapy of septic inhibitors of
inflammatory shock, cachexia, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis
and cytokines AIDS. The compounds are typically prepared by reaction of an iodo substituted
nucleoside with the appropriately

5. Document ID: US 6091708 A

L1: Entry 5 of 24 File: USPT Jul 18, 2000

US-PAT-NO: 6091708
DOCUMENT-IDENTIFIER: US 6091708 A

TITLE: Traffic shaper with multiply queued virtual paths

DATE-ISSUED: July 18, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Matsunuma; Keiji	Tokyo	N/A	N/A	JPX

US-CL-CURRENT: 370/233; 370/253, 370/397, 370/465

AB: A traffic shaper that feeds cells into an asynchronous-transfer-mode
virtual-path-switching network has two queues per virtual path: a first queue providing a
regular class of service, and a second queue providing a variable class of service. Cells are
taken from the first queue in preference to the second queue, combined traffic from the two
queues is kept within a peak cell rate, and traffic from the second queue is kept within an
allowed cell rate. Forward resource-management cells are inserted at certain intervals in the
cell flow taken from the second queue, and the allowed cell rate is adjusted according to
backward resource-management cells received in reply. A third queue for priority cell traffic,
limited to a priority cell rate lower than the peak cell rate, may also be provided.

L1: Entry 5 of 24 File: USPT Jul 18, 2000

DOCUMENT-IDENTIFIER: US 6091708 A
TITLE: Traffic shaper with multiply queued virtual paths

DEPR:
Referring to FIG. 9, the priority queue 70 is controlled with reference to a priority cell rate
parameter (EPCR) and a corresponding flag (FE) stored in the traffic control table 32. Each virtual
path has a separate EPCR value and FE flag. The EPCR value is less than the peak cell rate PCR and,
like the PCR value, can be set by software.

DEPR:
FIG. 10 shows the internal structure of the output scheduler 30 in the second embodiment, using the
same reference numerals as in FIG. 3 for corresponding elements. The queue flag register 56 now
stores three flag bits per virtual path, the QR and QV flags indicating the presence of cells in the

regular-class queue 22 and variable-class queue 24, and the QE flag indicating the presence of cells
in the priority queue 70. The EPCR controller 72 in FIG. 10 manages the FE time-out flags according
to the EPCR parameters, in the same way that the PCR controller 48 manages the FR flags according to
the PCR parameters.

DEPR:
The operation of the second embodiment is illustrated in FIG. 11. A certain amount (EPCR) of the
total bandwidth (PCR) of each virtual path is allocated to cells in the priority class (C=2). The
rate at which priority cells are sent cannot exceed the limit set by the EPCR parameter. The part of
the total bandwidth (PCR) not used by priority cell traffic is allocated to cells in the regular
class (C=1). Any bandwidth remaining after that is allocated to cells in the variable class (C=0)
and to FRM cells.

6. Document ID: US 6037450 A

L1: Entry 6 of 24
File: USPT
Mar 14, 2000

US-PAT-NO: 6037450
DOCUMENT-IDENTIFIER: US 6037450 A

TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

DATE-ISSUED: March 14, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Esmon; Charles T.	Oklahoma City	OK	N/A	N/A
Stearns-Kurosawa; Deborah J.	Edmond	OK	N/A	N/A
Kurosawa; Shinichiro	Edmond	OK	N/A	N/A

US-CL-CURRENT: 530/350; 530/827, 530/830

AB: Plasma EPCR has been isolated, characterized and shown to block cellular protein C
activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and
circulates at approximately 100 ng/ml (98.4.+-.27.8 ng/ml, n=22). Plasma EPCR bound activated
protein C with an affinity similar to that of recombinant soluble EPCR (Kd.sub.app
approximately 30 nM), and inhibits both protein C activation on an endothelial cell line and
APC anticoagulant activity in a one-stage factor Xa clotting assay. Soluble plasma EPCR appears
to attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant
function of activated protein C. Soluble EPCR has also been detected in urine. Levels of
soluble EPCR can rise in inflammatory disease associated with vascular injury and appear to be
correlated with inflammation and disease states associated with abnormal coagulation. Since
EPCR expression is restricted to larger vessels and is usually negative in cappillaries, these
observations provide a mechanism for analyzing injury/stimulation of large vessel endothelial
cells.

L1: Entry 6 of 24
File: USPT
Mar 14, 2000

DOCUMENT-IDENTIFIER: US 6037450 A
TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

ABPL:
Plasma EPCR has been isolated, characterized and shown to block cellular protein C activation and
APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at
approximately 100 ng/ml (98.4.+-.27.8 ng/ml, n=22). Plasma EPCR bound activated protein C with an
affinity similar to that of recombinant soluble EPCR (Kd.sub.app approximately 30 nM), and inhibits
both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage
factor Xa clotting assay. Soluble plasma EPCR appears to attenuate the membrane-bound EPCR
augmentation of protein C activation and the anticoagulant function of activated protein C. Soluble
EPCR has also been detected in urine. Levels of soluble EPCR can rise in inflammatory disease
associated with vascular injury and appear to be correlated with inflammation and disease states
associated with abnormal coagulation. Since EPCR expression is restricted to larger vessels and is
usually negative in cappillaries, these observations provide a mechanism for analyzing
injury/stimulation of large vessel endothelial cells.

BSPR:
Endothelial cells play a critical role in the protein C pathway in that they express two of the
known receptors responsible for efficient protein C activation, thrombomodulin and the endothelial
protein C/APC receptor (EPCR) (Fukudome and Esmon. 1994. J. Biol. Chem. 269:26486-26491;
Stearns-Kurosawa, et al. 1996. Proc. Natl. Acad. Sci. (USA) 93:10212-10216). Thrombomodulin (CD141)
is a transmembrane cofactor that binds circulating thrombin with high affinity and the resultant
enzyme-cofactor complex is required for physiologically relevant protein C activation rates (Esmon
and Owen. 1981. Proc. Natl. Acad. Sci. (USA) 78:2249-2252; Dittman, W. A. 1991. Trends Cardiovasc.
Med. 1:331-336).

BSPR:
EPCR is a recently identified receptor with significant homology to the CD1/MHC class 1 family
(Fukudome and Esmon, 1994; Fukudome, et al. 1996. J. Biol. Chem. 271:17491-17498; Regan, et al.
1996. J. Biol. Chem. 271:17499-17503). The cloning and biological role of the endothelial cell
receptor for protein C was described in PCT/US95/09636 by Oklahoma Medical Research Foundation,
entitled "Cloning and Regulation of an Endothelial Cell Protein

C/Activated Protein C Receptor". The
protein was predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence
at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a
type I transmembrane protein.

BSPR:
EPCR binds both protein C and APC with similar affinity (Kd.sub.app .about.30 nM) (Fukudome, et al.,
1996) in the presence of calcium and facilitates protein C activation by presenting the protein C
substrate to the thrombin-thrombomodulin activation complex on cell surfaces (Stearns-Kurosawa, et
al., 1996). Both endothelial cell receptors are type I transmembrane proteins in which the ligand
binds to an extracellular domain and both have a short intracellular cytoplasmic tail (Fukudome, et
al. 1996; Jackman, et al. 1987. Proc. Natl. Acad. Sci. (USA) 84:6425-6429; Wen, et al., 1987.
Biochemistry 26:4350-4357; Suzuki, et al. 1987. EMBO J. 6:1891-1897). In addition, their in vitro
cell surface expression is down-regulated similarly by tumor necrosis factor-.alpha.(Fukudome and
Esmon 1994). However, the characteristics of soluble forms of thrombomodulin and EPCR differ in
several respects. Recombinant soluble thrombomodulin has reduced cofactor activity relative to the
membrane form (Galvin, et al. 1987. J. Biol. Chem. 262:2199-2205; Parkinson, et al. 1990. J. Biol.
Chem. 265:12602-12610). With both purified components and with cells, the changes in thrombin's
substrate specifically induced by thrombomodulin result from competition for a shared binding domain
on thrombin as well as conformational alterations in the active site pocket (Ye, et al. 1991. J.
Biol. Chem. 266:23016-23021; Lu, et al. 1989. J. Biol. Chem. 264:12956-12962; Ye, et al. 1992. J.
Biol. Chem. 267:11023-11028; Hofsteenge, et al. 1986. Biochem. J. 237:243-251; Mathews, 1994.
Biochemistry 33:13547-13552; Esmon, et al. 1982. J. Biol. Chem. 257:7944-7947; Sadler, et al. 1993.
Haemostasis 23:183-193). Soluble thrombomodulin also accelerates inactivation of thrombin by a
variety of inhibitors (Bourin and Lindahl. 1993. Biochem. J. 289:313-330; Rezaie, 1995. J. Biol.
Chem. 270:25336-25339). Both plasma and urine contain detectable thrombomodulin (Takano, et al.
1990. Blood. 76:2024-2029; Ishii and Majerus. 1985. J. Clin. Invest. 76:2178-2181) and because the
thrombomodulin gene does not contain introns (Jackman, et al., 1987), these soluble forms are due to
proteolysis of the extracellular domain at the cell surface.

BSPR:
In contrast, recombinant soluble EPCR (rsEPCR), truncated just before the transmembrane domain,
binds both protein C and APC with an affinity similar to that observed for intact cell-surface
expressed EPCR (Fukudome, et al. 1996). APC anticoagulant activity is inhibited effectively when
bound to rsEPCR (Regan, et al., 1996), presumably because both rsEPCR and factor Va share binding
determinants in a groove reminiscent of the anion binding exosite I in thrombin occupied by
thrombomodulin (Mather, et al. 1996. EMBO J. 15:6822-6831). However, rsEPCR does not appear to
influence proteolysis of small synthetic substrates by APC, nor inactivation of APC by
.alpha.1-antitrypsin or protein C inhibitor (Regan, et al., 1996). Unlike membrane-bound EPCR which
enhances protein C activation (Stearns-Kurosaw, at al., 1996), rsEPCR has little effect on protein C
activation by the soluble thrombin-thrombomodulin complex (Regan, et al., 1996), suggesting that any
soluble forms of EPCR might inhibit protein C activation by competing with membrane-associated EPCR
for protein C.

BSPR:
Immunohistochemistry indicates that EPCR is present primarily on the surface of endothelial cells
from large vessels and is absent or present at low levels on most capillary endothelial cells.

BSPR:
It is therefore an object of the present invention to identify therapeutic and diagnostic uses for
naturally occurring soluble EPCR.

BSPR:
It is a further object of the present invention to characterize naturally occurring soluble EPCR.

BSPR:
Plasma EPCR (has been isolated, characterized and shown to block cellular protein C activation and
APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at
approximately 100 ng/ml (98.4.+-.27.8 ng/ml, n=22). Plasma EPCR was purified from human citrated
plasma using ion-exchange, immunoaffinity, and protein C affinity chromatography. Flow cytometry
experiments demonstrated that plasma EPCR bound activated protein C with an affinity similar to that
previously determined from recombinant truncated EPCR (Kd.sub.app approximately 30 nM), defined as
EPCR not including the transmembrane and cytoplasmic domains. Furthermore, plasma EPCR inhibited
both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage
factor Xa clotting assay. Soluble EPCR has also been detected in human urine. Cloning of the gene
encoding EPCR demonstrates that at least human EPCR can be alternatively spliced, yielding a
truncated soluble EPCR including an insert unique to the alternatively spliced form (sEPCR). These
results indicate that plasma EPCR can be derived either by proteolysis at the cell surface or by
alternative splicing.

BSPR:
If the local concentrations of plasma EPCR are sufficiently high, particularly in disease states,
the data indicates that the truncated soluble plasma EPCR could attenuate the membrane-bound EPCR
augmentation of protein C activation and the anticoagulant function of activated protein C. As
demonstrated by the examples comparing normal plasma EPCR with levels of EPCR from patients with an
autoimmune disease (systemic lupus erythematosus, SLE) and sepsis (a disorder involving both
inflammation and coagulation abnormalities), levels of soluble EPCR appear to be correlated with
inflammation and disease states associated with abnormal coagulation. Assays are described based on
measurement of soluble EPCR which are indicative of disease conditions involving coagulation,
inflammation, and large vessel disease. Assay reagents are described, including isolated purified
soluble EPCR, recombinant truncated soluble EPCR, and antibodies to the soluble EPCRs.

DRPR:
FIG. 1 is a schematic of the two known mechanisms for producing a soluble receptor as applied to
EPCR, by proteolysis of the membrane-bound receptor to release an extracellular domain and leave the
membrane anchor behind, and by alternative splicing of the mRNA, showing the sequences unique to
membrane bound EPCR (mEPCR) and to proteolyzed plasma EPCR (pEPCR), and the sequence unique to
soluble EPCR (sEPCR).

DRPR:
FIG. 3 shows the sequence inserted into human, bovine, murine, and baboon EPCR by alternative

splicing.

DRPR:
FIG. 4a is a graph showing that soluble plasma EPCR binds to human protein C and APC. EA.hy926 cells were incubated with 60 nM fl-APC in the presence of 0-500 nM rsEPCR (.circle-solid.) or plasma EPCR (.smallcircle.) for 30 minutes on ice. The cells were washed and cell-bound fluorescence was determined by flow cytometry as described. The intrinsic cell fluorescence in the absence of added fl-APC is indicated by the arrow. The mean cell fluorescence (MCF) plotted represents the average of duplicate MCF determinations.

DRPR:
FIGS. 4b and 4c are graphs showing soluble plasma EPCR and rsEPCR inhibit protein C activation on cell surfaces. In FIG. 4b, EA.hy926 cell monolayers were pre-incubated for 15 minutes at room temperature with 0.1 .mu.M protein C alone (.quadrature.) or with 1 .mu.M rsEPCR (.circle-solid.), or 2 .mu.g/ml 1494 mAb (.smallcircle.). Protein C activation was initiated by the addition of thrombin (2 nM final) and the reactions were stopped at the indicated times. Activated protein C was determined with an amidolytic assay and the activity rates in mOD/min are plotted for each time point. Control wells without added thrombin were included (.circle-solid.). Each data point represents the average of triplicate well determinations. In FIG. 4c, EA.hy926 cell monolayers were pre-incubated for 15 minutes at room temperature with 0.1 M protein C and the indicated concentrations of plasma EPCR (.smallcircle.) or rsEPCR (.circle-solid.). Thrombin (final 2 nM) was added and the activation proceeded for 60 minutes at room temperature. The supernatants were added to a mixture of antithrombin and heparin and activated protein C activities (mOD/min) were determined with an amidolytic assay. Each data point represents the average of triplicate well determinations.

DRPR:
FIG. 4d is a graph showing soluble plasma EPCR inhibits APC anticoagulant activity The anticoagulant activity of APC (25 nM) was determined with a one-stage Xa clotting assay in the presence of 460 nM plasma EPCR or rsEPCR. The effect was reversed when either soluble EPCR was pre-incubated for 5 minutes with 42 .mu.g/ml of 1496 mAb which blocks binding of APC to EPCR. The data represent the average of 4-6 determinations .+-.S.D.

DRPR:
FIG. 5 is a graph comparing levels of soluble plasma TM to soluble plasma PCR in lupus patients, demonstrating that there is no correlation between TM and EPCR values, but that the majority of lupus patients exhibit extremely elevated levels of soluble plasma EPCR.

DEPR:
Endothelial Protein C Receptor, EPCR.

DEPR:
Previous investigations into the function of EPCR found that protein C binding to the membrane form of EPCR resulted in facilitation of protein C activation by the thrombin-thrombomodulin complex on cell surfaces (Stearns-Kurosawa, et al., 1996), but that soluble recombinant EPCR inhibited APC anticoagulant activity (Regan, et al. 1996). These observations, along with the knowledge that soluble thrombomodulin degradation products in plasma are a marker of endothelial damage in various disease states, led to the question of whether a soluble circulating form(s) of EPCR existed and, if so, what role it may have in the protein C pathway.

DEPR:
The examples demonstrate that a soluble form of EPCR circulates in plasma and is present in urine.
In a healthy donor population, the plasma EPCR level was about 100 ng/ml and it appeared to be a single antigen species of approximately 43,000 daltons. Subsequent purification of the soluble EPCR from plasma and functional studies determined that it was capable of binding both protein C and APC with an affinity similar to intact membrane-bound EPCR. The in vitro studies using an endothelial cell line demonstrated that plasma EPCR inhibited protein C activation at near physiological concentrations of protein C and thrombin. In addition, direct addition of purified plasma EPCR to plasma resulted in inhibition of APC anticoagulant activity that was reversed with monoclonal antibodies to rsEPCR.

DEPR:
The identification of the purified plasma protein as being EPCR was based on comparison with the properties of rsEPCR. These proteins both reacted with the same battery of monoclonal and polyclonal antibodies, had the same amino-terminal sequence, bound to immobilized protein C in a Ca.sup.2+ -dependent fashion, and blocked protein C activation and APC anticoagulant activity with similar dose response curves. In addition, the affinities of both protein C and APC for rsEPCR and plasma EPCR are similar to the affinity of intact membrane-bound EPCR. These properties appear to be unique to EPCR.

DEPR:
Previous studies demonstrated that membrane-bound EPCR expressed on endothelial cells augments protein C activation by a factor of between three and five fold, whereas the examples demonstrate that the soluble form of EPCR purified from plasma inhibits protein C activation on endothelial cells and APC anticoagulant activity. This predicts that EPCR could modulate the protein C pathway in several ways. First, in the larger vessels where thrombomodulin concentration is low to the microcirculation, EPCR expression is correspondingly increased (Laszik, et al., Circulation 1997).
Immunohistochemistry shows that in most organs, EPCR expression is most intense on large vessels and decreases progressively with decreasing vessel size, with little or no expression in the most abundant endothelial cell type, the capillary endothelium. EPCR expression may play a critical role in capturing the protein C substrate from the circulation and presenting it to the thrombin-thrombomodulin complex for activation. This is supported by in vitro observations that both the EA.hy926 endothelial cell line and human umbilical vein endothelial cells have at least six times more surface-expressed EPCR antigen than thrombomodulin. In the microcirculation where thrombomodulin concentration is high and EPCR is low, one would predict little influence on protein C activation. Finally, circulating soluble EPCR may reduce the generation of APC and the ability of APC to inactivate factor Va.

DEPR:
In a healthy individual, the soluble EPCR levels are about 2.5 nM, a concentration well below both the Kd.sub.app (approximately 30 nM) and the 80 nM protein C concentration in the circulation. Both of the effects of soluble plasma EPCR (inhibition of APC anticoagulant activity and protein C activation) required considerably higher concentrations than that present in normal plasma, leaving

the question of the physiological role of the plasma EPCR uncertain. Patients with soluble EPCR
levels that exceed 40 nM have been identified, as described in Example 3 (lupus). Thus, if the local
concentration near the endothelial cell surface exceeds the systemic concentration, the soluble EPCR
concentration would reach levels that would attenuate both APC generation and activity, contributing
to thrombotic risk.

DEPR:
A soluble form of a receptor can be produced by proteolytic cleavage of the membrane-bound receptor
or by alternative splicing mechanisms. Proteolysis at the membrane surface releases soluble
thrombomodulin, and receptors for TNF, IL-1, IL-2, M-CSF, PDGF, and NGF (Heaney, et al. 1996. Blood
87:847-857). Soluble receptors have a multitude of potential functions including acting as
antagonists of the membrane receptor, stabilizing the ligand, initiating ligand-mediated signaling,
downmodulation of the membrane form, and binding to receptor inhibitors to indirectly facilitate
receptor-ligand activity. The latter mechanism is used by the IL-1 receptor system in which the
soluble isoforms of both IL-1 receptors are generated by proteolytic cleavage and tightly regulate
the responsiveness to IL-1.alpha. and IL-1.beta. (Arend, et al. 1994. J. Immunol. 153:4766-4774).
The EPCR genomic structure contains an alternative splicing site which would code for a soluble
protein truncated just before the transmembrane domain (Fukudome and Esmon. 1995. J. Biol. Chem.
270:5571-5577), as discussed below. Soluble IL-6 receptors appear to be generated by both
proteolytic and alternative splicing mechanisms (Mullberg, et al. 1994. J. Immunol. 152:4958-4968;
Lust, et al. 1992. Cytokine 4:96-100; Horiuchi, et al. 1994. Eur. J. Immunol. 24:1945-1948). This
cleavage site can also be useful in recovering large quantities of soluble EPCR, by constructing an
expression vector encoding the truncated EPCR immediately followed by a peptide sequence to which an
antibody is specifically directed, as described in U.S. Pat. No. 5,298,599 to Morrissey and Esmon,
the teachings of which are incorporated herein. The epitope will then be cleaved by proteolysis,
before or after administration to a patient. See also U.S. Pat. No.4,782,137 to Hopp et al.

DEPR:
Immunohistochemical studies have indicated that EPCR is located primarily on endothelium of large
vessels and is barely detectable in capillaries. Plasma EPCR derived from membrane-bound EPCR, can
therefore serve as a marker of large vessel disease processes. Plasma EPCR may serve as a useful
comparison with plasma thrombomodulin levels which have been shown to be modulated in a variety of
disease states, but which would reflect both large and small vessel disease processes, but probably
would be dominated by small vessel contributions since most endothelium is microvascular.

DEPR:
The cDNA for EPCR is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which
includes a 15 amino acid signal sequence (von Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at
the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Direct
sequencing of the recombinant protein showed that the mature protein started at Ser18. Sequence ID
No. 2 is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2
(MLTTLLPILLLSGWA) are the putative signal sequence determined by the method of von Heijne (von
Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2
(LVLGVLVGGFIIAGVAVGIFLCTGGR) are the
putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49,
64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at
amino acids 17 (removed in plasma EPCR), 114, 118, and 186 of Sequence ID No. 2. A potential
transmembrane region (Engelman et al., (1986) Annu. Rev. Biophys. Chem. 15, 321-53) consisting of 23
amino acids was identified at the C-terminal end (beginning at amino acid 211 of Sequence ID No. 2).

DEPR:
The protein is a type 1 transmembrane protein. The extracellular domain contains four potential
N-glycosylation sites and three Cys residues. Glycosylation is not essential for activity, as shown
by N-glycanase digestion. The cytoplasmic region contains only three amino acids and terminates with
a Cys, which is palmitoylated. If the terminal cysteine is not properly palmitoylated, the protein
may be secreted. Altering the sequence of the EPCR to replace this cysteine with another amino acid
thereby provides a means for making an essentially full length EPCR which is secreted instead of
being membrane bound.

DEPR:
Details of the following studies and results are described in the examples. Human plasma contains
about 100 ng/ml of soluble EPCR (Table 1). This was measured by an enzyme linked immunoassay (ELISA)
using two monoclonal antibodies (1494 mAb and 1495 mAb) and standard techniques. Significantly
elevated soluble EPCR levels were found in patients with systemic lupus erythematosus and sepsis.
These levels seemed fairly high for a membrane-bound receptor that is present, with few exceptions,
only on the surface of the large blood vessels. To put this in perspective, thrombomodulin (TM) is
expressed on all endothelium, as well as some non-vascular cells, yet normal soluble TM levels are
only about 10-40 ng/ml (Takano, et al., Blood 76:2024-2029, 1990). The soluble TM levels were
elevated in the patients with lupus, but not sepsis. Importantly, there was no correlation between
the plasma EPCR and TM levels in these patient groups (r.sup.2 =0.028 and 0.034, respectively).

DEPR:
The lack of correlation between the plasma EPCR and TM levels and the high plasma EPCR concentration
is consistent with the concept that plasma EPCR originates from both proteolytic and alternative
splicing mechanisms. The genomic structure of human EPCR contains four exons, separated by introns.
Review of this sequence reveals an in-frame reading sequence after the exon III--intron III boundary
(at the 5' GT) that includes a TAA stop codon at position 7527. Since this stop codon is upstream of
exon IV that codes for the transmembrane domain, the predicted protein would contain a unique 48
residue carboxyl-terminal tail (coded for by the intron sequence) and would not contain a
transmembrane anchor.

DEPR:
FIG. 1 is a diagram of two potential ways truncated EPCR can be derived: by proteolysis immediately
before the transmembrane domain or by alternative splicing. As shown by FIG. 2, alternative splicing
results in inclusion of a peptide sequence in the alternatively spliced truncated EPCR. As shown by
FIG. 3, this sequence is highly conserved between species, although slight differences exist,
resulting in a new carboxyl-terminal tail of 48 residues for human and bovine EPCR, 51 residues for

murine EPCR, and 22 residues for baboon EPCR.

DEPR:
Patient samples can be screened for the presence of, and amount of, sEPCR or EPCR, using antibodies to either EPCR, the unique insert present in the alternatively spliced insert in EPCR, or antibodies which bind with greater affinity to either EPCR or sEPCR due to conformational differences. Samples can also be screened using other standard techniques to specifically quantitate proteins which are present.

DEPR:
Antibodies to EPCR, and in particular, soluble EPCR ("sEPCR"), and recombinant soluble EPCR ("rsEPCR") can be generated which are useful in detection, characterization or isolation of receptor proteins, as well as for modifying receptor protein activity, in most cases, through inhibition of ligand binding. Antibodies are generated by standard techniques, using human or animal purified or recombinant receptor proteins or fragments thereof as the immunogen.

DEPR:
Monoclonal antibodies to EPCR were obtained as described for other proteins by Esmon, et al., 1993. Methods Enzymol. 222:359-385. The antibodies referred to as 1494, 1495, and 1496 mAbs are IgG1.kappa. antibodies that bind to recombinant soluble EPCR and to cell surface-expressed EPCR. The 1494 and 1496 mabs block the binding of protein C and APC to EPCR, and inhibit the ability of cellular EPCR to facilitate protein C activation by the thrombin-thrombomodulin complex. The 1495 mAb does not block ligand binding to EPCR, does not alter cell surface protein C activation, and has a binding epitope distinct from that for 1494 or 1486 mAb. The antibodies can be labelled using standard techniques, such as radiolabelling, enzyme labelling, fluorescent labels such as fluorescein, gold particles, dyes, and other means for detection of the antibodies. For example, antibody can be biotinylated with biotinamidocaproate N-hydroxysuccinimide ester using standard procedures. Antibody can be immobilized to a solid support for use in immunoassays, for example, AffiGel-10.TM., nitrocellulose, or microtiter wells, or use in solution phase immunoassays.

DEPR:
In a preferred embodiment, EPCR is measured using microtiter plates (Maxisorp.TM., NUNC NS, Roskilde, Denmark) coated with 50 microliters of 4 micrograms/ml 1495 mAb in 15 mM Na.sub.2 CO.sub.3, 35 mM NaHCO.sub.3, pH 9.6, at 4.degree. C. overnight. At room temperature, the plates are then washed three times with 20 mM Tris-HCl, 0.1 M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer), and blocked with assay buffer containing 0.1% (wt/vol) gelatin for at least one hour. The wells are then washed, 50 microliter samples added in triplicate wells, and the plates incubated for one hour. The wells are aspirated, washed three times with assay buffer, and 50 microliters of 2 micrograms/ml biotin-1494 mAb added. The plates are incubated for 1 hour, washed three times, and 50 microliters of 0.25 micrograms/ml streptavidin-alkaline phosphatase conjugate (GIBCO BRL) added and incubated for an additional hour. The wells are washed five times, and the substrate and amplifier reagents from an ELISA amplification kit (GIBCO BRL) added sequentially at 15-min intervals according to the manufacturer's directions. The color development is stopped with 0.3 M H.sub.2 SO.sub.4, and the endpoint absorbance read at 490 nm on a V.sub.max microplate reader. Standards in triplicate wells are from 1.5 to 100 ng rsEPCR/ml in 20 mM Tris-HCl, 0.1 M NaCl, and 1 mM EDTA, 0.1% gelatin, pH 7.5.
The standard curve is linear from 1.5 to 12.5 ng/ml, and samples are diluted with the same buffer to fall within the linear range. Studies show that between one and two percent plasma does not affect the linearity of the assay or the sensitivity of the standard curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma, and EPCR antigen levels are calculated from the average of triplicate wells by reference to standard curve determined on the same plate.

DEPR:
The assay for soluble EPCR is useful in detection and analysis of coagulation and inflammatory states and disorders as discussed herein, such as autoimmune diseases like lupus, in transplant monitoring, sepsis, shock, pre-eclampsia, diabetes, cardiopulmonary bypass, unstable angina, restenosis, angioplasty (i.e., vascular disease), kidney or liver disease. For example, the EPCR is a marker for large blood vessels, and therefore for damage to large blood vessels. An increase in the amount of soluble EPCR is indicative of large vessel injury, resulting either in proteolysis of EPCR or stimulation of sEPCR synthesis. The ratio of EPCR to thrombomodulin can also be determined, based on either blood or urine samples, which is indicative of the relative extent of microvascular versus large vessel. The relative amounts of EPCR to cytokines, leukocyte activation markers and complement factors or activation markers can also be used to indicate disease state.

DEPR:
Since EPCR is present on endothelial cells, it is useful as a marker of endothelial cell damage. It can be used as an indicator of drug effect, both toxicity as well as efficacy. For example, in lupus patients, drugs effectively minimizing inflammatory/coagulation mediated, large vessel injury would result in decreasing EPCR levels.

DEPR:
The following abbreviations are used: rsEPCR, recombinant soluble EPCR with the HPC4 epitope inserted in place of the transmembrane domain and cytosolic tail; mAb, monoclonal antibody; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

DEPR:
An alternative assay was developed in which the coating and detecting antibodies were reversed (1494 mAb coating; biotin-1495 mAb detecting) and antibody binding was detected with the Blue Phos substrate (KPL Laboratories; Gaithersburg, Md.). This method was used to assay plasma EPCR in the sepsis patients. This assay was more sensitive, probably because of affinity differences, but both assays gave qualitatively similar results.

DEPR:
In the first preparation, plasma (1L) was diluted with an equal volume of 20 mM Tris-HCl, pH 7.5, 10 mM benzamidine, 400 units sodium heparin and batch-adsorbed for 1 hour with 1 g pre-swollen QAE resin. After settling, the resin was processed for purification of protein C (Esmon, et al. 1993).
Solid ammonium sulfate was added to the supernatant at 4.degree. C. to 40% saturation, centrifuged, and additional ammonium sulfate was added to that supernatant to achieve 70% saturation. After centrifugation, the soft pellet was placed in dialysis bags and dialyzed overnight against 12 L of 20 mM Tris-HCl, 0.02% sodium azide, pH 7.4. The dialysate was applied to a 1496 mAb-AffiGel-10 immunoaffinity column (6 ml resin; 5 mg IgG/ml resin) equilibrated in 20

mM Tris-HCl, 0.1 M NaCl,
0.02% sodium azide, pH 7.4. The column was washed with more than 12 ml of the same buffer and eluted
with 50% (v/v) ethylene glycol in 20 mM Tris-HCl, pH 7.4 (Jun Xu, unpublished observations). The
peak fractions from the elution were pooled (0.37 total OD.sub.280), concentrated (Centriprep 30,
Millipore), and the buffer exchanged to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM CaCl.sub.2, 0.6 mM
MgCl.sub.2, 0.02% sodium azide, pH 7.4. This material was applied to a protein C affinity column
that had been previously prepared by applying the purified protein C (3 mg) to an HPC4-AffiGel-10
column (5 mg IgG/ml resin; 0.9.times.8 cm) in the same buffer. The HPC4 mAb binds the protein C
activation region in a calcium-dependent fashion (Esmon, et al. 1993; Stearns, et al. 1988) and does
not interfere with subsequent binding of EPCR to protein C. After applying the sample containing
plasma EPCR, the column was washed with approximately 12 ml of buffer and eluted with 20 mM
Tris-HCl, 0.1 M NaCl 5 mM EDTA, 10 mM MOPS, 0.02% sodium azide, pH 7.5. Fractions were monitored for
absorbance at 280 nm and for EPCR antigen using the ELISA described above. The eluate containing
both protein C and plasma EPCR was applied to an FPLC (Pharmacia-LKB, Uppsala, Sweden) Mono Q column
and the column developed with a linear gradient of 0.1-1 M NaCl in 20 mM Tris-HCl, pH 7.5. About
half of the plasma EPCR did not bind to the Mono Q column, half eluted at about 0.2 M NaCl, and the
protein C eluted at approximately 0.5 M NaCl. Both ionic species of plasma EPCR appeared identical
on SDS-PAGE gels under reducing or non-reducing conditions with silver staining, with Coomassie BB
staining, or with gold staining (Pierce) after transfer to PVDF membranes, and on Western blots with
the biotin-polyclonal anti-rsEPCR antibody probe.

DEPR:
The second preparation of plasma EPCR was done starting with 4L of plasma to purify enough protein
for functional studies. In this case, the 1496-AffiGel-10 resin (20 ml of 5 mg IgG/ml resin) was
added directly to the citrated plasma, along with final concentrations of 10 mM benzamidine, 1 mM
diisopropylfluorophosphate, and 0.5 units/ml sodium heparin. The plasma was batch-adsorbed overnight
at 4.degree. C. with gentle mixing. After the resin settled, the supernatant was processed for
protein C purification (Esmon, et al. 1993). The resin was packed into a 2.5.times.30 cm column,
washed extensively with 20 mM Tris-HCl, 0.1 M NaCl, 0.02% sodium azide, pH 74 and eluted with 50%
ethylene glycol in 20 mM Tris-HCl, pH 7.4. The eluate was pooled and concentrated (5.5 total
OD.sub.280), applied to a Mono Q column and the two ionic species (breakthrough and 0.2 M NaCl
eluate peak) were re-applied to the 1496-AffiGel-10 resin (1.5.times.11 cm). The column was eluted
with 50% ethylene glycol as before. The eluate (0.71 ODs) was concentrated and the buffer exchanged
to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, 0.02% sodium azide with a
Centriprep 30. This material was then applied to an affinity column in which protein C (2.9 mg) had
been initially applied in the same buffer to an HPC2-AffiGel-10 column (0.6.times.17 cm). The HPC2
mAb binds to the protein C serine protease domain and does not interfere with EPCR binding
(Fukudome, et al. 1996). The bound EPCR was eluted with buffer containing 5 mM EDTA. Contaminating
serum amyloid P (from the protein C sample) was removed by ion-exchange chromatography on the FPLC
Mono Q column. The sample was applied in 0.2 M NaCl, so that the plasma EPCR did not bind, and was
separated from the contaminants which eluted at 0.4-0.5 M NaCl. The resultant purified plasma EPCR
(0.193 OD.sub.280) appeared homogenous by SDS-PAGE with silver staining and by Western blotting with
polyclonal anti-rsEPCR. This material was used for the functional studies and amino-terminal
sequence analysis.

DEPR:
Plasma EPCR purity was determined from silver stained SDS-PAGE 10% gels and western blots of
membranes probed with biotin-goat anti-rsEPCR (reduced and non-reduced). A single band of
approximately 43,000 Da appears in both the serum and plasma samples after the membrane is probed
with the polyclonal antibody. The size of the protein detected appears slightly larger than the
rsEPCR. The other bands detected were background binding of IgG as judged by probing with preimmune
IgG and longer exposure times. overnight incubation of plasma samples with the anti-EPCR 1495 mAb
coupled to AffiGel-10 resin, followed by washing and elution of bound antigen under reducing
conditions, resulted in a single band detected by Western blotting with biotin-goat anti-rsEPCR
polyclonal antibody.

DEPR:
Determination of soluble EPCR antigen in plasma from healthy volunteers by ELISA using mAb 1495 as
the coating antibody found antigen levels of 91.1+/-24.5 ng/ml in females (n=12) and 107.2+/-30.2
ng/ml in males (n=10). When calculated together, the average plasma EPCR antigen level was
98.4+/-27.8 ng/ml. The value for males appeared to be slightly higher than for females, similar to
thrombomodulin (Quehenberger, et al. Thromb. Haemost. 76: 729-734), although the population studied
was too limited for statistical analysis and this study was not designed to assess differences due
to gender, age, diet or other variables.

DEPR:
Since the plasma EPCR appeared to be a single species at approximately 100 ng/ml, it became
important to determine whether the circulating EPCR could bind protein C and APC. Soluble EPCR was
purified from human plasma by a combination of ion-exchange chromatography, precipitation with
ammonium sulfate, and immunoadsorption by anti-EPCR 1496 mAb-AffiGel-10 column chromatography as
described in Experimental Procedures.

DEPR:
This plasma EPCR (approximately 110 .mu.g) was applied to a protein C affinity column prepared by
applying protein C (3 mg) to an anti-protein C HPC4 mAb-AffiGel-10 column in buffer containing 3 mM
CaCl.sub.2, 0.6 mM MgCl.sub.2. The column was washed and plasma EPCR was applied at fraction 19. The
column was washed and eluted with buffer containing 5 mM EDTA starting at fraction 35. Absorbance at
280 nm and EPCR antigen was determined for the fractions. EPCR antigen was determined by ELISA.

DEPR:
More than 98% of the applied plasma EPCR antigen bound to the protein C affinity column. The
absorbance profile indicates co-elution of EPCR and protein C from the antibody column, consistent
with the calcium-dependence of protein C binding to this antibody (Stearns, et al. 1988).

DEPR:
To purify sufficient protein for functional and structural studies, EPCR was purified from 4L of
plasma using a similar, but slightly modified procedure. After elution from a protein C-antibody
affinity column, residual contaminating proteins were removed by ion-exchange chromatography on an

FPLC Mono Q column. The resultant preparation of plasma EPCR appeared homogenous on SDS-PAGE 10%
gels with silver staining and identical results were obtained with western blots probed with
biotin-goat anti-rsEPCR polyclonal antibody under both reducing and non-reducing conditions.
Amino-terminal sequence analysis of the purified protein yielded only one sequence, S-Q-D-A-S-D,
which is identical to the amino-terminal sequence of recombinant soluble EPCR (Sequence ID No. 2).
This is the first amino-terminal sequence determination of EPCR from a natural source.

DEPR:
The ability of plasma EPCR to bind to APC was assessed by competition studies in which plasma EPCR
was allowed to compete with cellular EPCR for APC, and the resultant free APC that could bind to
cellular EPCR was assessed by flow cytometry (FIG. 4a). APC labeled with fluorescein in the active
site (fl-APC) was incubated with EA.hy926 cells in the presence or absence of either plasma EPCR or
rsEPCR. The EPCR concentration dependence for inhibition of APC binding to the cells was similar for
both soluble forms of EPCR. This observation indicates that the affinity of plasma EPCR for binding
APC is similar to that previously determined for the rsEPCR-APC binding interaction (Kd.sub.app
approximately 30 nM).

DEPR:
While rsEPCR has little effect on protein C activation in a soluble system (Regan, et al. 1996),
membrane-bound EPCR has a very potent ability to facilitate activation on cell surfaces
(Stearns-Kurosawa, et al. 1996). The current data demonstrating the existence of a circulating form
of EPCR capable of binding protein C and APC suggested that plasma EPCR has the potential to alter
cell-surface activation of protein C. The thrombin-dependent activation of an approximately
physiological level of protein C (0.1 .mu.M) on EA.hy926 cells was inhibited by excess rsEPCR almost
to the level of that observed with the anti-rsEPCR 1494 mAb that blocks the EPCR-protein C binding
interaction, as shown by FIG. 4b. Previous studies have demonstrated that rsEPCR has no effect on
APC amidolytic activity using small synthetic substrates (Regan, et al. 1996). The plasma EPCR was
slightly more effective in its ability to inhibit cell-surface protein C activation on the EA.hy926
cells relative to the rsEPCR, as shown by FIG. 4c.

DEPR:
In a one-stage factor Xa clotting assay, purified plasma and soluble recombinant EPCR inhibited the
APC prolongation of clotting times similarly (FIG. 4d). Inhibition of APC anticoagulant activity by
rsEPCR had been observed previously (Regan, et al. 1996). As expected, the 1496 mAb reversed this
effect by blocking the APC-plasma EPCR binding interaction.

DEPR:
To address the question of whether soluble EPCR is present in urine, four urine samples were
collected (first morning void) and analyzed for the presence of soluble EPCR by western blotting and
ELISA.

DEPR:
Undiluted pediatric urine samples were compared to a 4% normal plasma and recombinant soluble EPCR
(1 ng). The samples were incubated with biotin-goat-anti-rsEPCR and a streptavidin-alkaline
phosphatase detection system.

DEPR:
The western blot indicates that a) soluble EPCR is present in urine, and b) the soluble EPCR antigen
is present at a size similar to that observed in plasma. Obvious degradation is not observed. The
amount of soluble EPCR in the four samples as quantified by ELISA was 40.3, 6.1, 35.6, and 90.1
ng/ml.

DEPR:
Normal human plasma EPCR concentration are about 100 ng/ml (98.4.+-.27.8 ng/ml; 2.5 nM), as
discussed above. A panel of samples from patients with lupus erythematosus (n=54) was assayed and
soluble EPCR levels were found to range from non-detectable levels to greater than 1,700 ng/ml.
Fifteen patients had soluble EPCR levels greater than 200 ng/ml.

DEPR:
Previous studies have shown elevated soluble plasma TM levels in lupus patients due to endothelial
damage and the current lupus patient samples were assayed for plasma TM as a reference. It was found
that their soluble TM levels had absolutely no correlation with their soluble EPCR levels, as shown
by FIG. 5. This is an important observation that suggests that the source of the soluble plasma EPCR
is not simply from randomly damaged endothelium. In contrast to TM, membrane-bound EPCR expression
in humans and primates is restricted primarily to the endothelium of large vessels, with capillaries
expressing little EPCR. The distinctive localization of EPCR is expected to augment protein C
activation locally to prevent large vessel thrombosis. The primary localization of membrane-bound
EPCR to the large vessels points to a targeted thrombotic risk in the large vessels that may be
predicted by soluble plasma EPCR concentrations.

DEPR:
Patient blood samples were taken at time 0 (entry into the Intensive Care Unit, ICU) and at two days
and six days after treatment with anti-thrombin II (ATIII) or a placebo. Plasma soluble EPCR and
soluble thrombomodulin (TM) were assayed only on time 0 samples.

DEPR:
These results are shown graphically in FIG. 6. As in the lupus patients, patients with sepsis show
very significant elevations in plasma EPCR levels, not correlated with soluble TM levels.

DEPR:
The observation that soluble plasma EPCR inhibits both protein C activation and activated protein C
anticoagulant activity indicates that the elevated plasma EPCR levels in these patients poses an
additional thrombotic risk and marks evidence of vascular injury/responsiveness. Examples of
conditions these are indicative of include disorders associated with endothelial cell stimulation,
atherogenesis, leukocyte adhesion and plaque rupture.

DEPR:
As an initial approach to determine whether a soluble EPCR isoform could be generated by alternative
splicing mechanism, RNA was isolated from human and baboon tissues and reverse transcriptase-PCR
(RT-PCR) performed with gene-specific primers. Although the baboon EPCR genomic sequence is not
known, primers based on the human sequence were used based on the reasoning that baboons and humans
are closely related on the evolutionary scale.

DEPR:
In the RT-PCR procedure generally, total RNA is isolated from homogenized tissue. The RNA is mixed
with a specific antisense primer, nucleotides and the reverse transcriptase enzyme. In the mix, the
RNA serves as a template for the reverse transcriptase to create a first

strand cDNA. This new cDNA
template is then amplified by conventional PCR using specific primers and Taq polymerase. Primers
that would amplify both the membrane form of EPCR (424 bp) and the predicted alternatively spliced
product (674 bp) were chosen. Products corresponding to both forms of EPCR were amplified from a
variety of baboon tissues (FIG. 4) and human lung and placenta. Possible contamination with genomic
DNA was unlikely as judged by controls without reverse transcriptase and the lack of a 1,885 bp band
in the reactions with the tissues.

DEPR:
The observation that the predicted soluble EPCR isoforms will have unique carboxyl terminal tails
provides a structural difference for distinguishing between the isoforms using isoform-specific
antibodies. The working model is that plasma levels of proteolyzed soluble EPCR will report
endothelial injury, whereas levels of alternatively spliced soluble EPCR will report an endothelial
response to stimuli. It is anticipated that the relative plasma levels of the soluble EPCR isoforms
will provide information on large vessel endothelial dysfunction and injury in specific pathologies.

DEPR:
RT-PCR products from human tissues: placenta, lung, and tongue, were electrophoresed using the
CRES/CREA primers specific for EPCR. The procedures were the same as used for the baboon tissues.
Products corresponding to the membrane isoform of EPCR (mEPCR) and the alternatively-spliced soluble
EPCR isoform (sEPCR) were observed. The products look essentially the same as that seen using the
baboon tissues. The only difference is that the placental tissue appears to have additional
products.

DEPL:
Detection and Characterization of Soluble EPCR; Physiological Role and Utility as a Marker

DEPL:
Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPL:
Proteins. Human protein C (Esmon, et al. 1993. Methods Enzymol. 222:359-385), bovine thrombin (Owen,
et al. 1974. J. Biol. Chem. 249:594-605), and bovine antithrombin (Esmon 1977. "Factors regulating
the inhibition of thrombin by antithrombin III. In Chemistry and Biology of Thrombin". R. L.
Lundblad, J. W. Fenton, II, and K. G. Mann, editors. Ann Arbor Science, Ann Arbor. 403-411) were
purified as described. Recombinant soluble EPCR, rsEPCR, consists of the extracellular domain of
EPCR truncated at residue 210 just before the transmembrane domain, followed by a 12 residue
sequence that permits calcium-dependent immunoaffinity purification on the HPC4 monoclonal antibody
(Takahashi, et al. 1992; Stearns, et al. 1988. J. Biol. Chem. 263:826-832). The construction,
purification, and protein C/APC binding characteristics of rsEPCR (Fukudome, et al. 1996). Goat
preimmune serum and polyclonal antiserum to rsEPCR was prepared and the IgG purified (Fukudome, et
al. 1996). Goat anti-rsEPCR polyclonal antibody was biotinylated with biotinamidocaproate
N-hydroxysuccinimide ester using standard procedures.

DEPL:
Monoclonal antibodies. Monoclonal antibodies (mAb) against rsEPCR were obtained as described for
other proteins (Esmon, et al. 1993). The 1494, 1495, and 1496 mAb are IgG1k antibodies that bind to
rsEPCR and to cell surface-expressed EPCR. The 1494 and 1496 mAb block the binding of protein C and
APC to EPCR and inhibit the ability of cellular EPCR to facilitate protein C activation by the
thrombin-thrombomodulin complex (Stearns-Kurosawa, et al. 1996). The 1495 mAb does not block ligand
binding to EPCR, does not alter cell surface protein C activation and has a binding epitope distinct
from that for 1494 or 1496 mAb. The 1494 and 1495 mAbs were biotinylated with biotinamidocaproate
N-hydroxysuccinimide ester using standard procedures. The 1494 mAb was coupled to AffiGel-10,
according to the manufacturer's directions, for immunoaffinity purification of plasma EPCR. The
screening of anti-EPCR mAb was done using methods described by Stearns-Kurosawa, et al. (1996);
Fukudome, et al. (1996).

DEPL:
Clotting Assay. The effect of rsEPCR or purified plasma EPCR on APC (25 nM) anticoagulant activity
in a one-stage factor Xa clotting assay was performed (Regan, et al. 1996) in the presence or
absence of 83 .mu.g/ml 1496 mAb, an antibody that blocks APC-EPCR interaction (Stearns-Kurosawa, et
al. 1996). The soluble EPCRs and 1496 mAb were pre-incubated for 15 minutes before assay.

DEPL:
Flow Cytometric Analysis. To serve as a fluorescent probe, APC was labeled with fluorescein in the
active site (fl-APC) as described (Fukudome and Esmon, 1994; Bock, P. E. 1988. Biochemistry
27:6633-6639). The effect of rsEPCR or plasma EPCR on APC binding to EA.hy926 cells was studied by
flow cytometry (Fukudome, et al. 1996). Briefly, harvested cells were incubated for 30 min on ice
with 60 nM fl-APC in the absence or presence of increasing concentrations of either soluble EPCR
preparation, washed, and cell-bound fluorescence was determined by flow cytometry with 10,000 events
counted per sample. All assays were done in Hank's balanced salt solution supplemented with 1%
bovine serum albumin, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, and 0.02% sodium azide.

DEPL:
Cell surface protein C activation. EA.hy926 cells were cultured in 96-well tissue culture dishes
(Stearns-Kurosawa, et al. 1996). The confluent monolayers were washed three times with Hank's
balanced salt solution supplemented with 1% (w/v) bovine serum albumin, 3 mM CaCl.sub.2, 0.6 mM
MgCl.sub.2, and 0.02% sodium azide. All assays were done at room temperature in the same buffer in
60 .mu.l final volume, and all protein concentrations represent the final concentration in the
assay. Protein C was added (0.1 .mu.M) in the absence or presence of rsEPCR, plasma EPCR, or 1494
mAb at the indicated concentrations and pre-incubated with the cells for 15 minutes. Thrombin was
added to the mixtures (2 nM) to start the activation reactions. At the indicated time, 50 .mu.l
aliquots were removed and added to 10 .mu.l of antithrombin (0.7 .mu.M final) and heparin (5 U/ml
final) in a 96-well microtiter plate. APC amidolytic activity was determined by addition of
Spectrozyme PCa substrate (0.2 mM) and the rate of change in absorbance at 405 nm (mOD/min) was
measured on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, Calif.). All assay
points were done in triplicate wells and less than 10% of the protein C substrate was activated as
determined by reference to a standard curve of fully activated protein C versus mOD/min.

DEPL:
ELISA for quantitation of plasma EPCR. An enzyme-linked

immunosorbent assay for detection of EPCR
antigen in plasma was developed. Microtitre plates (Maxisorp; Nunc, Roskilde, Denmark) were coated
with 50 .mu.l of 4 .mu.g/ml 1495 mAb in 15 mM Na.sub.2 CO.sub.3, 35 mM NaHCO.sub.3, pH 9.6 at
4.degree. C. overnight. The following steps were done at room temperature. The wells were washed
three times with 20 mM Tris-HCl, 0.1 M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer) and blocked with
assay buffer containing 0.1% (w/v) gelatin for at least one hour. The wells were washed, 50 .mu.l
samples were added in triplicate wells, and the plates were incubated for one hour. The wells were
aspirated, washed three times with assay buffer and 50 .mu.l of 2 .mu.g/ml biotin-1494 mAb was
added. The plates were incubated for one hour, washed three times and 50 .mu.l of 0.25 .mu.g/ml
streptavidin-alkaline phosphatase conjugate (GibcoBRL) was added and incubated for an additional one
hour. The wells were washed five times and the substrate and amplifier reagents from an ELISA
amplification kit (GibcoBRL) were added sequentially at 15 minute intervals according to the
manufacturer's directions. The color development was stopped with 0.3 M H.sub.2 SO.sub.4 and the
endpoint absorbance at 490 nm was read on a Vmax microplate reader. Each plate contained standards
in triplicate wells from 1.5-100 ng/ml rsEPCR in 20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 0.1%
gelatin, pH 7.5. The standard curve was linear (r=0.99) from 1.5-12.5 ng/ml and plasma samples were
diluted with the same buffer to fall within the linear range. Preliminary experiments determined
that a final concentration of 1-2% human plasma did not affect the linearity or sensitivity of the
standard curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1
mM EDTA to a final 2% plasma and EPCR antigen levels were calculated from the average of triplicate
wells by reference to a standard curve determined on the same plate.

DEPL:
Purification of plasma EPCR. Plasma EPCR as purified from human citrated plasma (Oklahoma Blood
Institute) using a combination of ion-exchange chromatography, anti-rsEPCR mAb immunoaffinity
chromatography, and chromatography on protein C affinity columns. Two preparations were done in
slightly different ways.

DEPL:
Protein Sequencing. The amino-terminal sequence analysis of soluble plasma EPCR was performed in Dr.
Kenneth Jackson's laboratory at the Molecular Biology Research Facility, W. K. Warren Medical
Research Institute, Oklahoma City. Amino acids are designated by the standard one letter code.

DEPL:
A. EPCR ELISA: The coating antibody is 1494 mAb that binds to the ligand binding domain of EPCR. The
detecting antibody is biotinylated 1495 mAb, which does not block protein C/APC binding, and does
not cross-react with 1494 mAb. The detection system is streptavidin-alkaline phosphatase and
BluePhos substrate (from KPL).

DEPC:
Identification of Functional Endothelial Protein C Receptor in Human Plasma

DEPC:
Detection of Soluble EPCR in Urine

DEPC:
Measurement of Plasma EPCR from Lupus Patients

DEPC:
Plasma Soluble EPCR in Septic Shock Patients

DEPC:
Identification of Alternatively Spliced Forms of EPCR in Baboon and Human Tissues

DETL:
TABLE 1 _____ Plasma soluble receptor levels Plasma EPCR Plasma TM
ng/ml ng/ml _____ Normal volunteers, 133.4 .+-. 53.4 °35.5 .+-.
20.4 n = 20 Systemic lupus 262.1 .+-. 154.5° 104.7 .+-. 77.5° erythematosus, (P = 0.0004) (P =
0.0008) n = 40 Sepsis, n = 24 224.9 .+-. 74.5° 39.9 .+-. 73.1 (P = 0.00009)
_____ °Significant difference between the means relative to normal;
unpaired Student's t test.

CLPR:
1. A modified endothelial protein C receptor having the structure of the protein defined by Seq. ID
No. 2, residues 16-238 wherein the carboxyl terminal cysteine residue is replaced with another amino
acid or is not palmitoylated.

CLPR:
2. The modified endothelial protein C receptor of claim 1 which is not glycosylated.

CLPR:
3. The modified endothelial protein C receptor of claim 1 further comprising a signal sequence
having the structure of Seq. ID No. 2, residues 1-15.

CLPR:
4. The modified endothelial protein C receptor of claim 1 encoded by Seq. ID No. 1, or a nucleotide
sequence hybridizing thereto under stringent conditions.

CLPR:
5. The modified endothelial protein C receptor of claim 4 encoded by Seq. ID No. 1.

CLPR:
6. The modified endothelial protein C receptor of claim 1 wherein the carboxyl terminal cysteine
residue is replaced with another amino acid.

CLPR:
7. The modified endothelial protein C receptor of claim 1 wherein the carboxyl terminal cysteine
residue is not palmitoylated.

CLPR:
8. The modified endothelial protein C receptor of claim 1 which is human endothelial protein C
receptor except for the modification.

CLPR:
9. An isolated naturally occurring alternatively spliced endothelial protein C receptor present in
plasma having the structure defined by Seq. ID No. 2, further comprising a protein insert after Gly
201.

CLPR:
10. The isolated naturally occurring alternatively spliced endothelial protein C receptor of claim 9
comprising protein inserts as shown in FIG. 3.

CLPR:
11. An isolated naturally occurring soluble endothelial protein C receptor having the structure of
Seq. ID No. 2, residues 16-201, wherein the receptor has been cleaved at a naturally occuring
proteolytic cleavage site before the transmembrane domain present in plasma.

CLPR:
12. The isolated naturally occurring soluble endothelial protein C receptor of claim 11 encoded by a
DNA sequence hybridizing to Seq. ID No. 1 under stringent conditions.

ORPL:
Kurosawa, et al., "Identification of functional Endothelial Protein C Receptor in Human Plasma," J.
Clin. Invest. 1000(2): 411-418 (1997).

ORPL:
Fukudome and Esmon, "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein
C/Activated Protein C Receptor (EPCR)--The Structural and Functional Conservation in Human, Bovine
and Murine EPCR*," J. Biol. Chem. 270(10):5571-5577 (1995).

7. Document ID: US 5852171 A

L1: Entry 7 of 24 File: USPT Dec 22, 1998

US-PAT-NO: 5852171
DOCUMENT-IDENTIFIER: US 5852171 A

TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

DATE-ISSUED: December 22, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Fukudome; Kenji	Oklahoma City	OK	N/A	N/A
Esmon; Charles T.	Oklahoma City	OK	N/A	N/A

US-CL-CURRENT: 530/350; 530/380

AB: Human protein C and activated protein C were shown to bind to endothelium
specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca.sup.2+
dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I
transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of
the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation,
the endothelial cell protein C receptor (EPCR) function and message are both down regulated by
exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily
provides insights into the role of protein C in regulating the inflammatory response, and
determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 7 of 24 File: USPT Dec 22, 1998

DOCUMENT-IDENTIFIER: US 5852171 A
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ABPL:
Human protein C and activated protein C were shown to bind to endothelium specifically, selectively
and saturably (Kd=30 nM, 7000 sites per cell) in a Ca.sup.2+ dependent fashion. Expression cloning
revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding
protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the
receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function
and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a
member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the
inflammatory response, and determination of methods for pharmaceutical use in manipulating the
inflammatory response.

BSPR:
An endothelial cell protein C binding protein (referred to herein as "EPCR") has been cloned and
characterized. The protein is predicted to consist of 238 amino acids, which includes a 15 amino
acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes
the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein
C and activated protein C (Kd=30 nM) and is calcium dependent. The message and binding function of
the receptor are both down regulated by cytokines such as TNF.

BSPR:
These results identify a new member of a complex pathway that, like other members of the pathway, is
subject to regulation by inflammatory cytokines, and can therefore be used to modulate inflammatory
reactions in which protein C or activated protein C is involved. Inhibition of the inflammatory
response can be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in
contact with blood will render the surfaces anticoagulant by virtue of the ability of EPCR to bind
and concentrate the anticoagulant activated protein C at the surface. Alternatively, the function of
EPCR can be enhanced by overexpressing the EPCR in endothelium that could be used to coat vascular
grafts in patients with vascular disease or on stents in cardiac patients.

DRPR:
FIGS. 3A and 3B are flow cytometric analyses of F1-APC binding to 293T cells transfected with a cDNA
clone of EPCR. Cells were transfected with a clone EPCR/pEF-BOS or pEF-BOS (negative control) by the
calcium/phosphate method. After 24 h, cells were harvested and F1-APC binding was performed in the
absence (dotted lines) or presence of 1.3 mM CaCl2 (solid lines).

DRPR:
FIG. 4 is the predicted protein structure of EPCR based on nucleotide sequence, predicted amino acid
sequence and a hydropathy plot of EPCR. The signal sequence and transmembrane region are indicated
with the solid bars.

DRPR:
FIG. 5 is a comparison of the amino acid sequence of EPCR to the amino acid sequences of other

members of the CD1 family and CCD41. The EPCR sequence is shown in the first line and compared to
murine CCD41 (second line). human CD1d (third line) and murine CD1.2 (fourth line). Identities with
EPCR are indicated by open boxes. Residues that are conserved between EPCR and all of the human CD1
family members are indicated by a double asterisk. Residues shared with one or more members of the
CD1 family are indicated by a single asterisk.

DRPR:
FIG. 6 is a comparison of the amino acid sequence of human EPCR (first line) to the amino acid
sequence of murine EPCR (second line). Identities are indicated by boxes. Similarities are indicated
with an asterisk.

DEPR:
Human protein C and activated protein C are shown to bind to endothelium specifically, selectively
and saturably (Kd=30 nM, 7000 sites per cell) in a Ca.sup.2+ dependent fashion. FL-APC binding to
various human cell lines were examined, and found that the binding was HUVEC specific. A human
kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these
binding sites. A HUVEC cDNA library was constructed using the powerful mammalian expression vector,
PEF-BOS (Mizushima and Nagata, (1990) Nucleic Acids Res. 18, 5322). Plasmid DNA was prepared from
subpools of independent colonies (2,500 colonies per pool), and transfected into 293T cells, using
the method of Kaisho et al., (1994) Proc. Natl. Acad. Sci. (U.S.A.) 91, 5325. FL-APC binding was
analyzed on a flow cytometer. One of eight subpools gave a positive signal. This subpool was divided
into 20 subpools and rescreened. After three rounds of enrichment, one positive clone, EPCR-1, was
isolated. EPCR-1 carries a 1.3 kb insert. When transfected into 293T cells, this clone was capable
of expressing the calcium-dependent binding site for FL-APC on the 293T cell surface.

DEPR:
Expression cloning revealed a 1.3 kb cDNA that coded for a type I transmembrane glycoprotein capable
of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like
thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C
receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF.
Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of
this receptor for protein C in regulating the inflammatory response.

DEPR:
Total RNAs (15 .mu.g) from various cells were isolated, electrophoresed through formaldehyde agarose
gels and transferred to a nylon membrane (Hybond-N.TM., Amersham). The 483 bp Xba I fragment from
the 5' end of the EPCR cDNA was labeled by random priming according to the manufacturer's
instructions (Multiprime.TM. DNA labeling system, Amersham) and used for hybridization.

DEPR:
Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPR:
The cDNA is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which includes a
15 amino acid signal sequence (von Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at the
N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Sequence ID No. 2
is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2
(MLTTLLPILLLSGWA) are the putative signal sequence determined by the method of von Heijne (von
Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVLGVLVGGFIIAGVAVGIFLCTGGR) are the
putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49,
64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at
amino acids 17, 114, 118, and 186 of Sequence ID No. 2. A potential transmembrane region (Engelman
et al., (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 321-53) consisting of 23 amino acids was
identified at the C-terminal end (beginning at amino acid 211 of Sequence ID No. 2).

DEPR:
DNA and protein database searches revealed that the sequence is related to the
centrosome-associated, cell cycle dependent murine protein, CCD41, also referred as centrocyclin
(Rothbarth et al., (1993) J. Cell Sci. 104, 19-30), as shown by FIG. 5. The similarity in the
published sequence of murine CCD41 with human EPCR led to the cloning and sequencing of the murine
EPCR. The sequence of murine EPCR is shown in FIG. 6. It is distinct from the published sequence of
CCD41.

DEPR:
The EPCR amino acid sequence was also related to, but quite distinct from, the CD1/MHC superfamily
and the murine CD1.2, as also shown by FIG. 5. Based on the homology to CD1/MHC, it is likely that
EPCR contains two domains consisting of residues 17-114 and 118-188. Of the CD1 family members, CD1d
is the most similar to EPCR. In the mouse, CCD41 is associated exclusively with the centrosome
during G.sub.1 but becomes detectable elsewhere during the cell cycle, reaching a maximum during
G.sub.2, except during the G.sub.2 /M phase (Rothbarth et al., 1993). EPCR expression appears
restricted to endothelium, which would not be expected for a cell cycle associated protein.

DEPR:
The identification of the protein C receptor on endothelium suggests that the endothelial cell binds
protein C/APC through three distinct mechanisms. In addition to EPCR, protein S can bind APC/protein
C on negatively charged membrane surfaces that include the endothelium (Stern et al., (1986) J.
Biol. Chem. 261, 713-718), but this is not cell type specific (Dahlback et al., 1992).
Thrombomodulin in complex with thrombin can bind protein C and APC (Hogg et al., 1992). On
endothelium, the protein S binding sites (Nawroth and Stern, (1986) J. Exp. Med. 163, 740-745),
thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that
inflammation can impair protein C pathway function at multiple levels.

DEPR:
The homology to the CD.sup.1 /MHC family of proteins is especially interesting since it provides
indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed
.alpha.1,2 and 3. The extracellular domain of EPCR contains four Cys residues that appear to
correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two
domains have significant homology to the .alpha.1 and .alpha.2 domains of the CD1 protein family and
the .alpha.2 domain of the MHC class 1 protein, suggesting that these proteins evolved from a common
ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites
and is rich in .beta. strand structure, suggesting that it may form a .beta. sheet. Despite the

.beta. strand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6,
381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR,
residues 118-188, contains two additional N glycosylation sites and, like the CD1/MHC family, this
domain is predicted to have limited .beta. structure.

DEPR:
In vitro studies have suggested anti-inflammatory activities for APC. For instance, an unusual
carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to selectins (Grinnell at
al., (1994) Glycobiology, 4, 221-226) Modest inhibitory effects of APC have been reported on TNF
production (Grey et al., (1993) Transplant. Proc. 25, 2913-2914). EPCR could contribute to these
anti-inflammatory mechanisms. Since the homologous protein family, CD1, can be linked to CD8
(Ledbetter et al., (1985) J. Immunol. 134, 4250-4254), it is also possible that the proteins C
receptor is linked to another protein and signal through this second protein. One of the CD1 family
members, CD1d, has been reported to promote T cell responses, possibly involving binding to CD8
(Panja et al., (1993) J. Exp. Med. 178, 1115-1119). CD1b has recently been reported to serve as an
antigen presenting molecule (Porcelli et al., (1992) Nature 360, 593-597). The ability to bind
protein C/APC could then be linked either directly or indirectly to signalling via direct
interaction with cells of the immune system. Since the MHC class of proteins is involved in
presentation of proteins to cell receptors, the concept of presentation of protein C/APC to
inflammatory cells as a means of elaborating anti-inflammatory activity may also be involved. This
includes modulation of enzyme specificity such as occurs with thrombin-thrombomodulin interaction
(Esmon, 1989). In this case, the EPCR-APC complex might cleave biologically active peptides from
unknown substrates.

DEPR:
EPCR mRNA Levels and APC Binding

DEPR:
To determine the cellular specificity of EPCR expression, the intensity of FL-APC binding to HUVEC
was compared to several human cell lines. Fl-APC bound strongly only to HUVEC, and not to any of the
T, B, or monocytic cell lines tested. Cells were incubated at room temperature without or with 160
nM F1-APC in the presence of 1.3 mM CaCl.sub.2. Binding was analyzed by flow cytometry. Slight
binding was demonstrated with the osteosarcoma line, HOS and the epidermoid carcinoma cell line,
HEp-2.

DEPR:
Total RNA was extracted from these cells and hybridized with the EPCR cDNA probe for Northern Blot
Analysis. EPCR mRNA was detected by Northern blot analysis for HUVEC, Jurkat, HEp-2, Raji, HOS, and
U937. Among the cells lines tested, EPCR mRNA was detected at high levels only in HUVEC. The
calculated mRNA size of 1.3 kb was identical to the size of the isolated cDNA. After prolonged
exposure, a weak signal was also detected with the osteosarcoma cell line HOS and monocyte cell line
U937. Thus, both APC binding and EPCR mRNA expression are very specific for endothelium.

DEPR:
Effects of TNF on APC Binding and EPCR MRNA Levels

DEPR:
Several other members of the protein C anticoagulant pathway are subject to regulation by
inflammatory cytokines (Esmon, 1989). For instance, endothelial cell surface thrombomodulin
expression and message are known to be reduced by exposure of the cells to TNF (Conway and
Rosenberg, 1988; Lentz et al., 1991). To determine if a similar process occurs with EPCR, HUVEC were
treated with TNF and APC binding and expression of EPCR mRNA were examined. APC binding to HUVEC
decreased in a time dependent fashion. EPCR activity decreased more rapidly than thrombomodulin
antigen. HUVEC were cultured for 0, 6, 24 and 48 hr, in the presence of TNF-.alpha. (10 ng/ml).
Cells were harvested and residual F1-APC binding or thrombomodulin (TM) expression was analyzed by
flow cytometry. Cell surface TM was stained with an anti-TM murine monoclonal antibody and
FITC-conjugated anti-mouse IgG. The negative control is without added fluorescent ligand.

DEPR:
HUVEC were treated with 10 ng/ml of TNF-.alpha. for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message
was extracted and detected as described above. The results demonstrated that the concentration of
EPCR mRNA was also reduced by TNF treatment. Message levels and APC binding activity decreased in
parallel. Therefore, the TNF mediated down-regulation of APC binding to endothelium probably occurs
at the level of mRNA expression.

DEPR:
Enhancement of inflammatory responses by blocking binding of endogenous molecules to ECPCR can be
achieved by administration of compounds binding to the receptor to a subject in need of inhibition.
The degree of binding is routinely determined using assays such as those described above. Compounds
which are effective include antibodies to the protein, fragments of antibodies retaining the binding
regions, and peptide fragments of APC which include the Gla region. Inhibition of the inflammatory
response could be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in
contact with blood would render the surfaces anticoagulant by virtue of the ability of EPCR to bind
and concentrate the anticoagulant APC at the surface. Alternatively, the function of EPCR could be
enhanced by overexpressing the EPCR in endothelium used to coat vascular grafts in patients with
vascular disease or on stents in cardiac patients.

DEPR:
Patients with thrombosis or hyperinflammatory conditions could be screened for defects in the EPCR
gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more
preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful
in this screening using hybridization assays of patient samples, including blood and tissues.
Screening can also be accomplished using antibodies, typically labelled with a fluorescent,
radiolabelled, or enzymatic label, or by isolation of target cells and screening for binding
activity, as described in the examples above. Typically, one would screen for expression on either a
qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with
.sup.32 P, .sup.35 S, fluorescein, biotin, or other labels routinely used with methods known to
those skilled in the art for labelling of proteins and/or nucleic acid sequences.

DEPR:
In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be
advantageous to increase its concentration in vivo on endothelium. The

binding assays described here
and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken
with thrombomodulin and investigators have shown that cyclic AMP (Maruyama, I. et al. (1991)
Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis, S. et al., (1991) Blood 78, 410-415)
can elevate thrombomodulin expression. The ability to screen such drugs or drugs that block TNF down
regulation of EPCR provide an approach to elevating EPCR expression in vivo and thus enhancing
anticoagulant and anti-inflammatory activity.

DEPR:
Studies based on inhibition of binding are predictive for indirect effects of alteration of receptor
binding. For example, inhibition of binding of APC or increased expression of TNF is predictive of
inhibition of EPCR function.

DEPR:
As described herein, a variety of compounds can be used to inhibit or enhance expression of the
EPCR. The nature of the disorder will determine if the expression should be enhanced or inhibited.
For example, based on the studies involving the use of an anti-protein C antibody in combination
with cytokine, it should be possible to treat solid tumors by enhancing an inflammatory response
involving blocking of protein C or activated protein C binding to an endothelial cell protein
C/activated protein C receptor by administering to a patient in need of treatment thereof an amount
of a compound blocking binding of protein C or activated protein C to the receptor. Similarly, it
should be possible to treat disorders such as gram negative sepsis, stroke, thrombosis, septic
shock, adult respiratory distress syndrome, and pulmonary emboli using a method for inhibiting an
inflammatory response involving administration of EPCR or EPCR fragments or substances that
upregulate EPCR expression to a patient in need of treatment therof.

DEPL:
I. Cloning and Characterization of EPCR.

DEPL:
II. Modulation of Inflammation using EPCR.

8. Document ID: US 5804392 A

L1: Entry 8 of 24 File: USPT Sep 8, 1998

US-PAT-NO: 5804392
DOCUMENT-IDENTIFIER: US 5804392 A

TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

DATE-ISSUED: September 8, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Esmon; Charles T.	Oklahoma City	OK	N/A	N/A
Stearns-Kurosawa; Deborah J.	Edmond	OK	N/A	N/A
Kurosawa; Shinichiro	Edmond	OK	N/A	N/A

US-CL-CURRENT: 435/7.1; 435/7.8, 435/975, 436/506, 530/387.1, 530/388.22, 530/389.1

AB: Plasma EPCR has been isolated, characterized and shown to block cellular protein C
activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and
circulates at approximately 100 ng/ml (98.4.+-.27.8 ng/ml, n=22). Plasma EPCR bound activated
protein C with an affinity similar to that of recombinant soluble EPCR (Kd.sub.app
approximately 30 nM), and inhibits both protein C activation on an endothelial cell line and
APC anticoagulant activity in a one-stage factor Xa clotting assay. Soluble plasma EPCR appears
to attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant
function of activated protein C. Soluble EPCR has also been detected in urine. Levels of
soluble EPCR can rise in inflammatory disease associated with vascular injury and appear to be
correlated with inflammation and disease states associated with abnormal coagulation. Since
EPCR expression is restricted to larger vessels and is usually negative in cappillaries, these
observations provide a mechanism for analyzing injury/stimulation of large vessel endothelial
cells.

L1: Entry 8 of 24 File: USPT Sep 8, 1998

DOCUMENT-IDENTIFIER: US 5804392 A
TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

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Plasma EPCR has been isolated, characterized and shown to block cellular protein C activation and
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affinity similar to that of recombinant soluble EPCR (Kd.sub.app approximately 30 nM), and inhibits
both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage
factor Xa clotting assay. Soluble plasma EPCR appears to attenuate the membrane-bound EPCR
augmentation of protein C activation and the anticoagulant function of activated protein C. Soluble
EPCR has also been detected in urine. Levels of soluble EPCR can rise in inflammatory disease
associated with vascular injury and appear to be correlated with inflammation and disease states
associated with abnormal coagulation. Since EPCR expression is restricted to larger vessels and is
usually negative in cappillaries, these observations provide a mechanism for analyzing

injury/stimulation of large vessel endothelial cells.

BSPR:
Endothelial cells play a critical role in the protein C pathway in that they express two of the
known receptors responsible for efficient protein C activation, thrombomodulin and the endothelial
protein C/APC receptor (EPCR) (Fukudome and Esmon. 1994. J. Biol. Chem. 269:26486-26491;
Stearns-Kurosawa, et al. 1996. Proc. Natl. Acad. Sci. (USA) 93:10212-10216). Thrombomodulin (CD141)
is a transmembrane cofactor that binds circulating thrombin with high affinity and the resultant
enzyme-cofactor complex is required for physiologically relevant protein C activation rates (Esmon
and Owen. 1981. Proc. Natl. Acad. Sci. (USA) 78:2249-2252; Dittman, W. A. 1991. Trends Cardiovasc.
Med. 1:331-336).

BSPR:
EPCR is a recently identified receptor with significant homology to the CD1/MHC class 1 family
(Fukudome and Esmon, 1994; Fukudome, et al. 1996. J. Biol. Chem. 271:17491-17498; Regan, et al.
1996. J. Biol. Chem. 271:17499-17503). The cloning and biological role of the endothelial cell
receptor for protein C was described in PCT/US95/09636 by Oklahoma Medical Research Foundation,
entitled "Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor". The
protein was predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence
at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a
type 1 transmembrane protein.

BSPR:
EPCR binds both protein C and APC with similar affinity (Kd.sub.app .about.30 nM) (Fukudome, et al.,
1996) in the presence of calcium and facilitates protein C activation by presenting the protein C
substrate to the thrombin-thrombomodulin activation complex on cell surfaces (Stearns-Kurosawa, et
al., 1996). Both endothelial cell receptors are type 1 transmembrane proteins in which the ligand
binds to an extracellular domain and both have a short intracellular cytoplasmic tail (Fukudome, et
al. 1996; Jackman, et al. 1987. Proc. Natl. Acad. Sci. (USA) 84:6425-6429; Wen, et al., 1987.
Biochemistry 26:4350-4357; Suzuki, et al. 1987. EMBO J. 6:1891-1897). In addition, their in vitro
cell surface expression is down-regulated similarly by tumor necrosis factor-.alpha. (Fukudome and
Esmon 1994). However, the characteristics of soluble forms of thrombomodulin and EPCR differ in
several respects. Recombinant soluble thrombomodulin has reduced cofactor activity relative to the
membrane form (Galvin, et al. 1987. J. Biol. Chem. 262:2199-2205; Parkinson, et al. 1990. J. Biol.
Chem. 265:12602-12610). With both purified components and with cells, the changes in thrombin's
substrate specifically induced by thrombomodulin result from competition for a shared binding domain
on thrombin as well as conformational alterations in the active site pocket (Ye, et al. 1991. J.
Biol. Chem. 266:23016-23021; Lu, et al. 1989. J. Biol. Chem. 264:12956-12962; Ye, et al. 1992. J.
Biol. Chem. 267:11023-11028; Hofsteenge, et al. 1986. Biochem. J. 237:243-251; Mathews, 1994.
Biochemistry 33:13547-13552; Esmon, et al. 1982. J. Biol. Chem. 257:7944-7947; Sadler, et al. 1993.
Haemostasis 23:183-193). Soluble thrombomodulin also accelerates inactivation of thrombin by a
variety of inhibitors (Bourin and Lindahl. 1993. Biochem. J. 289:313-330; Rezaie, 1995. J. Biol.
Chem. 270:25336-25339). Both plasma and urine contain detectable thrombomodulin (Takano, et al.
1990. Blood. 76:2024-2029; Ishii and Majerus. 1985. J. Clin. Invest. 76:2178-2181) and because the
thrombomodulin gene does not contain introns (Jackman, et al., 1987), these soluble forms are due to
proteolysis of the extracellular domain at the cell surface.

BSPR:
In contrast, recombinant soluble EPCR (rsEPCR), truncated just before the transmembrane domain,
binds both protein C and APC with an affinity similar to that observed for intact cell-surface
expressed EPCR (Fukudome, et al. 1996). APC anticoagulant activity is inhibited effectively when
bound to rsEPCR (Regan, et al., 1996), presumably because both rsEPCR and factor Va share binding
determinants in a groove reminiscent of the anion binding exosite I in thrombin occupied by
thrombomodulin (Mather, et al. 1996. EMBO J. 15:6822-6831). However, rsEPCR does not appear to
influence proteolysis of small synthetic substrates by APC, nor inactivation of APC by
.alpha.1-antitrypsin or protein C inhibitor (Regan, et al., 1996). Unlike membrane-bound EPCR which
enhances protein C activation (Stearns-Kurosaw, at al., 1996), rsEPCR has little effect on protein C
activation by the soluble thrombin-thrombomodulin complex (Regan, et al., 1996), suggesting that any
soluble forms of EPCR might inhibit protein C activation by competing with membrane-associated EPCR
for protein C.

BSPR:
Immunohistochemistry indicates that EPCR is present primarily on the surface of endothelial cells
from large vessels and is absent or present at low levels on most capillary endothelial cells.

BSPR:
It is therefore an object of the present invention to identify therapeutic and diagnostic uses for
naturally occurring soluble EPCR.

BSPR:
It is a further object of the present invention to characterize naturally occurring soluble EPCR.

BSPR:
Plasma EPCR (has been isolated, characterized and shown to block cellular protein C activation and
APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at
approximately 100 ng/ml (98.4.+-.27.8 ng/ml, n=22). Plasma EPCR was purified from human citrated
plasma using ion-exchange, immunoaffinity, and protein C affinity chromatography. Flow cytometry
experiments demonstrated that plasma EPCR bound activated protein C with an affinity similar to that
previously determined from recombinant truncated EPCR (Kd.sub.app approximately 30 nM), defined as
EPCR not including the transmembrane and cytoplasmic domains. Furthermore, plasma EPCR inhibited
both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage
factor Xa clotting assay. Soluble EPCR has also been detected in human urine. Cloning of the gene
encoding EPCR demonstrates that at least human EPCR can be alternatively spliced, yielding a
truncated soluble EPCR including an insert unique to the alternatively spliced form (sEPCR). These
results indicate that plasma EPCR can be derived either by proteolysis at the cell surface or by
alternative splicing.

BSPR:
If the local concentrations of plasma EPCR are sufficiently high, particularly in disease states,
the data indicates that the truncated soluble plasma EPCR could attenuate the membrane-bound EPCR
augmentation of protein C activation and the anticoagulant function of

activated protein C. As
demonstrated by the examples comparing normal plasma EPCR with levels of EPCR from patients with an
autoimmune disease (systemic lupus erythematosus, SLE) and sepsis (a disorder involving both
inflammation and coagulation abnormalities), levels of soluble EPCR appear to be correlated with
inflammation and disease states associated with abnormal coagulation. Assays are described based on
measurement of soluble EPCR which are indicative of disease conditions involving coagulation,
inflammation, and large vessel disease. Assay reagents are described, including isolated purified
soluble EPCR, recombinant truncated soluble EPCR, and antibodies to the soluble EPCRs.

DRPR:
FIG. 1 is a schematic of the two known mechanisms for producing a soluble receptor as applied to
EPCR, by proteolysis of the membrane-bound receptor to release an extracellular domain and leave the
membrane anchor behind, and by alternative splicing of the mRNA, showing the sequences unique to
membrane bound EPCR (mEPCR) and to proteolyzed plasma EPCR (pEPCR), and the sequence unique to
soluble EPCR (sEPCR).

DRPR:
FIG. 3 shows the sequence inserted into human, bovine, murine, and baboon EPCR by alternative
splicing.

DRPR:
FIG. 4a is a graph showing that soluble plasma EPCR binds to human protein C and APC. EA.hy926 cells
were incubated with 60 nM fl-APC in the presence of 0-500 nM rsEPCR (.circle-solid.) or plasma EPCR
(.largecircle.) for 30 minutes on ice. The cells were washed and cell-bound fluorescence was
determined by flow cytometry as described. The intrinsic cell fluorescence in the absence of added
fl-APC is indicated by the arrow. The mean cell fluorescence (MCF) plotted represents the average of
duplicate MCF determinations.

DRPR:
FIGS. 4b and 4c are graphs showing soluble plasma EPCR and rsEPCR inhibit protein C activation on
cell surfaces. In FIG. 4b, EA.hy926 cell monolayers were pre-incubated for 15 minutes at room
temperature with 0.1 .mu.M protein C alone (.quadrature.) or with 1 .mu.M rsEPCR (.circle-solid.),
or 2 .mu.g/ml 1494 mAb (.largecircle.). Protein C activation was initiated by the addition of
thrombin (2 nM final) and the reactions were stopped at the indicated times. Activated protein C was
determined with an amidolytic assay and the activity rates in mOD/min are plotted for each time
point. Control wells without added thrombin were included (.box-solid.). Each data point represents
the average of triplicate well determinations. In FIG. 4c, EA.hy926 cell monolayers were
pre-incubated for 15 minutes at room temperature with 0.1 .mu.M protein C and the indicated
concentrations of plasma EPCR (.largecircle.) or rsEPCR (.circle-solid.). Thrombin (final 2 nM) was
added and the activation proceeded for 60 minutes at room temperature. The supernatants were added
to a mixture of antithrombin and heparin and activated protein C activities (mOD/min) were
determined with an amidolytic assay. Each data point represents the average of triplicate well
determinations.

DRPR:
FIG. 4d is a graph showing soluble plasma EPCR inhibits APC anticoagulant activity The anticoagulant
activity of APC (25 nM) was determined with a one-stage Xa clotting assay in the presence of 460 nM
plasma EPCR or rsEPCR. The effect was reversed when either soluble EPCR was pre-incubated for 5
minutes with 42 .mu.g/ml of 1496 mAb which blocks binding of APC to EPCR. The data represent the
average of 4-6 determinations.+-.S.D.

DRPR:
FIG. 5 is a graph comparing levels of soluble plasma TM to soluble plasma PCR in lupus patients,
demonstrating that there is no correlation between TM and EPCR values, but that the majority of
lupus patients exhibit extremely elevated levels of soluble plasma EPCR.

DEPR:
Endothelial Protein C Receptor, EPCR.

DEPR:
Previous investigations into the function of EPCR found that protein C binding to the membrane form
of EPCR resulted in facilitation of protein C activation by the thrombin-thrombomodulin complex on
cell surfaces (Stearns-Kurosawa, et al., 1996), but that soluble recombinant EPCR inhibited APC
anticoagulant activity (Regan, et al. 1996). These observations, along with the knowledge that
soluble thrombomodulin degradation products in plasma are a marker of endothelial damage in various
disease states, led to the question of whether a soluble circulating form(s) of EPCR existed and, if
so, what role it may have in the protein C pathway.

DEPR:
The examples demonstrate that a soluble form of EPCR circulates in plasma and is present in urine.
In a healthy donor population, the plasma EPCR level was about 100 ng/ml and it appeared to be a
single antigen species of approximately 43,000 daltons. Subsequent purification of the soluble EPCR
from plasma and functional studies determined that it was capable of binding both protein C and APC
with an affinity similar to intact membrane-bound EPCR. The in vitro studies using an endothelial
cell line demonstrated that plasma EPCR inhibited protein C activation at near physiological
concentrations of protein C and thrombin. In addition, direct addition of purified plasma EPCR to
plasma resulted in inhibition of APC anticoagulant activity that was reversed with monoclonal
antibodies to rsEPCR.

DEPR:
The identification of the purified plasma protein as being EPCR was based on comparison with the
properties of rsEPCR. These proteins both reacted with the same battery of monoclonal and polyclonal
antibodies, had the same amino-terminal sequence, bound to immobilized protein C in a Ca.sup.2+
-dependent fashion, and blocked protein C activation and APC anticoagulant activity with similar
dose response curves. In addition, the affinities of both protein C and APC for rsEPCR and plasma
EPCR are similar to the affinity of intact membrane-bound EPCR. These properties appear to be unique
to EPCR.

DEPR:
Previous studies demonstrated that membrane-bound EPCR expressed on endothelial cells augments
protein C activation by a factor of between three and five fold, whereas the examples demonstrate
that the soluble form of EPCR purified from plasma inhibits protein C activation on endothelial
cells and APC anticoagulant activity. This predicts that EPCR could modulate the protein C pathway
in several ways. First, in the larger vessels where thrombomodulin concentration is low to the
microcirculation, EPCR expression is correspondingly increased (Laszik,

et al., Circulation 1997).

Immunohistochemistry shows that in most organs, EPCR expression is most intense on large vessels and decreases progressively with decreasing vessel size, with little or no expression in the most abundant endothelial cell type, the capillary endothelium. EPCR expression may play a critical role in capturing the protein C substrate from the circulation and presenting it to the thrombin-thrombomodulin complex for activation. This is supported by in vitro observations that both the EA.hy926 endothelial cell line and human umbilical vein endothelial cells have at least six times more surface-expressed EPCR antigen than thrombomodulin. In the microcirculation where thrombomodulin concentration is high and EPCR is low, one would predict little influence on protein C activation. Finally, circulating soluble EPCR may reduce the generation of APC and the ability of APC to inactivate factor Va.

DEPR:

In a healthy individual, the soluble EPCR levels are about 2.5 nM, a concentration well below both the Kd.sub.app (approximately 30 nM) and the 80 nM protein C concentration in the circulation. Both of the effects of soluble plasma EPCR (inhibition of APC anticoagulant activity and protein C activation) required considerably higher concentrations than that present in normal plasma, leaving the question of the physiological role of the plasma EPCR uncertain. Patients with soluble EPCR levels that exceed 40 nM have been identified, as described in Example 3 (lupus). Thus, if the local concentration near the endothelial cell surface exceeds the systemic concentration, the soluble EPCR concentration would reach levels that would attenuate both APC generation and activity, contributing to thrombotic risk.

DEPR:

A soluble form of a receptor can be produced by proteolytic cleavage of the membrane-bound receptor or by alternative splicing mechanisms. Proteolysis at the membrane surface releases soluble thrombomodulin, and receptors for TNF, IL-1, IL-2, M-CSF, PDGF, and NGF (Heaney, et al. 1996. Blood 87:847-857). Soluble receptors have a multitude of potential functions including acting as antagonists of the membrane receptor, stabilizing the ligand, initiating ligand-mediated signaling, downmodulation of the membrane form, and binding to receptor inhibitors to indirectly facilitate receptor-ligand activity. The latter mechanism is used by the IL-1 receptor system in which the soluble isoforms of both IL-1 receptors are generated by proteolytic cleavage and tightly regulate the responsiveness to IL-1.alpha. and IL-1.beta. (Arend, et al. 1994. J. Immunol. 153:4766-4774).

The EPCR genomic structure contains an alternative splicing site which would code for a soluble protein truncated just before the transmembrane domain (Fukudome and Esmon. 1995. J. Biol. Chem. 270:5571-5577), as discussed below. Soluble IL-6 receptors appear to be generated by both proteolytic and alternative splicing mechanisms (Mullberg, et al. 1994. J. Immunol. 152:4958-4968; Lust, et al. 1992. Cytokine 4:96-100; Horiuchi, et al. 1994. Eur. J. Immunol. 24:1945-1948). This cleavage site can also be useful in recovering large quantities of soluble EPCR, by constructing an expression vector encoding the truncated EPCR immediately followed by a peptide sequence to which an antibody is specifically directed, as described in U.S. Pat. No. 5,298,599 to Morrissey and Esmon, the teachings of which are incorporated herein. The epitope will then be cleaved by proteolysis, before or after administration to a patient. See also U.S. Pat. No.4,782,137 to Hopp et al.

DEPR:

Immunohistochemical studies have indicated that EPCR is located primarily on endothelium of large vessels and is barely detectable in capillaries. Plasma EPCR derived from membrane-bound EPCR, can therefore serve as a marker of large vessel disease processes. Plasma EPCR may serve as a useful comparison with plasma thrombomodulin levels which have been shown to be modulated in a variety of disease states, but which would reflect both large and small vessel disease processes, but probably would be dominated by small vessel contributions since most endothelium is microvascular.

DEPR:

The cDNA for EPCR is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which includes a 15 amino acid signal sequence (von Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Direct sequencing of the recombinant protein showed that the mature protein started at Ser18. Sequence ID No. 2 is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2 (MLTTLLPILLLSGWA) are the putative signal sequence determined by the method of von Heijne (von Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVLGVLVGGFIIAGVAVGIFLCTGGR) are the putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at amino acids 17 (removed in plasma EPCR), 114, 118, and 186 of Sequence ID No. 2. A potential transmembrane region (Engelman et al., (1986) Annu. Rev. Biophys. Chem. 15, 321-53) consisting of 23 amino acids was identified at the C-terminal end (beginning at amino acid 211 of Sequence ID No. 2).

DEPR:

The protein is a type 1 transmembrane protein. The extracellular domain contains four potential N-glycosylation sites and three Cys residues. Glycosylation is not essential for activity, as shown by N-glycanase digestion. The cytoplasmic region contains only three amino acids and terminates with a Cys, which is palmitoylated. If the terminal cysteine is not properly palmitoylated, the protein may be secreted. Altering the sequence of the EPCR to replace this cysteine with another amino acid thereby provides a means for making an essentially full length EPCR which is secreted instead of being membrane bound.

DEPR:

Details of the following studies and results are described in the examples. Human plasma contains about 100 ng/ml of soluble EPCR (Table 1). This was measured by an enzyme linked immunoassay (ELISA) using two monoclonal antibodies (1494 mAb and 1495 mAb) and standard techniques. Significantly elevated soluble EPCR levels were found in patients with systemic lupus erythematosus and sepsis.

These levels seemed fairly high for a membrane-bound receptor that is present, with few exceptions, only on the surface of the large blood vessels. To put this in perspective, thrombomodulin (TM) is expressed on all endothelium, as well as some non-vascular cells, yet normal soluble TM levels are only about 10-40 ng/ml (Takano, et al., Blood 76:2024-2029, 1990). The soluble TM levels were elevated in the patients with lupus, but not sepsis. Importantly, there was no correlation between the plasma EPCR and TM levels in these patient groups (r.sup.2 =0.028

and 0.034, respectively).

DEPR:
The lack of correlation between the plasma EPCR and TM levels and the high plasma EPCR concentration
is consistent with the concept that plasma EPCR originates from both proteolytic and alternative
splicing mechanisms. The genomic structure of human EPCR contains four exons, separated by introns.
Review of this sequence reveals an in-frame reading sequence after the exon III-intron III boundary
(at the 5' GT) that includes a TAA stop codon at position 7527. Since this stop codon is upstream of
exon IV that codes for the transmembrane domain, the predicted protein would contain a unique 48
residue carboxyl-terminal tail (coded for by the intron sequence) and would not contain a
transmembrane anchor.

DEPR:
FIG. 1 is a diagram of two potential ways truncated EPCR can be derived: by proteolysis immediately
before the transmembrane domain or by alternative splicing. As shown by FIG. 2, alternative splicing
results in inclusion of a peptide sequence in the alternatively spliced truncated EPCR. As shown by
FIG. 3, this sequence is highly conserved between species, although slight differences exist,
resulting in a new carboxyl-terminal tail of 48 residues for human and bovine EPCR, 51 residues for
murine EPCR, and 22 residues for baboon EPCR.

DEPR:
Patient samples can be screened for the presence of, and amount of, sEPCR or EPCR, using antibodies
to either EPCR, the unique insert present in the alternatively spliced insert in EPCR, or antibodies
which bind with greater affinity to either EPCR or sEPCR due to conformational differences. Samples
can also be screened using other standard techniques to specifically quantitate proteins which are
present.

DEPR:
Antibodies to EPCR, and in particular, soluble EPCR ("sEPCR"), and recombinant soluble EPCR
("rsEPCR") can be generated which are useful in detection, characterization or isolation of receptor
proteins, as well as for modifying receptor protein activity, in most cases, through inhibition of
ligand binding. Antibodies are generated by standard techniques, using human or animal purified or
recombinant receptor proteins or fragments thereof as the immunogen.

DEPR:
Monoclonal antibodies to EPCR were obtained as described for other proteins by Esmon, et al., 1993.
Methods Enzymol. 222:359-385. The antibodies referred to as 1494, 1495, and 1496 mAbs are IgGl.sub.K
antibodies that bind to recombinant soluble EPCR and to cell surface-expressed EPCR. The 1494 and
1496 mAbs block the binding of protein C and APC to EPCR, and inhibit the ability of cellular EPCR
to facilitate protein C activation by the thrombin-thrombomodulin complex. The 1495 mAb does not
block ligand binding to EPCR, does not alter cell surface protein C activation, and has a binding
epitope distinct from that for 1494 or 1486 mAb. The antibodies can be labelled using standard
techniques, such as radiolabelling, enzyme labelling, fluorescent labels such as fluorescein, gold
particles, dyes, and other means for detection of the antibodies. For example, antibody can be
biotinylated with biotinamidocaproate N-hydroxysuccinimide ester using standard procedures. Antibody
can be immobilized to a solid support for use in immunoassays, for example, AffiGel-10.TM.,
nitrocellulose, or microtiter wells, or use in solution phase immunoassays.

DEPR:
In a preferred embodiment, EPCR is measured using microtiter plates (Maxisorp.TM., NUNC NS,
Roskilde, Denmark) coated with 50 microliters of 4 micrograms/ml 1495 mAb in 15 mM Na.sub.2
CO.sub.3, 35 mM NaHCO.sub.3, pH 9.6, at 4.degree. C. overnight. At room temperature, the plates are
then washed three times with 20 mM Tris-HCl, 0.1M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer), and
blocked with assay buffer containing 0.1% (wt/vol) gelatin for at least one hour. The wells are then
washed, 50 microliter samples added in triplicate wells, and the plates incubated for one hour. The
wells are aspirated, washed three times with assay buffer, and 50 microliters of 2 micrograms/ml
biotin-1494 mAb added. The plates are incubated for 1 hour, washed three times, and 50 microliters
of 0.25 micrograms/ml streptavidin-alkaline phosphatase conjugate (GIBCO BRL) added and incubated
for an additional hour. The wells are washed five times, and the substrate and amplifier reagents
from an ELISA amplification kit (GIBCO BRL) added sequentially at 15-min intervals according to the
manufacturer's directions. The color development is stopped with 0.3M H.sub.2 SO.sub.4, and the
endpoint absorbance read at 490 nm on a V.sub.max microplate reader. Standards in triplicate wells
are from 1.5 to 100 ng rsEPCR/ml in 20 mM Tris-HCl, 0.1M NaCl, and 1 mM EDTA, 0.1% gelatin, pH 7.5.
The standard curve is linear from 1.5 to 12.5 ng/ml, and samples are diluted with the same buffer to
fall within the linear range. Studies show that between one and two percent plasma does not affect
the linearity of the assay or the sensitivity of the standard curve. Plasma samples from healthy
volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma, and EPCR
antigen levels are calculated from the average of triplicate wells by reference to standard curve
determined on the same plate.

DEPR:
The assay for soluble EPCR is useful in detection and analysis of coagulation and inflammatory
states and disorders as discussed herein, such as autoimmune diseases like lupus, in transplant
monitoring, sepsis, shock, pre-eclampsia, diabetes, cardiopulmonary bypass, unstable angina,
restenosis, angioplasty (i.e., vascular disease), kidney or liver disease. For example, the EPCR is
a marker for large blood vessels, and therefore for damage to large blood vessels. An increase in
the amount of soluble EPCR is indicative of large vessel injury, resulting either in proteolysis of
EPCR or stimulation of sEPCR synthesis. The ratio of EPCR to thrombomodulin can also be determined,
based on either blood or urine samples, which is indicative of the relative extent of microvascular
versus large vessel. The relative amounts of EPCR to cytokines, leukocyte activation markers and
complement factors or activation markers can also be used to indicate disease state.

DEPR:
Since EPCR is present on endothelial cells, it is useful as a marker of endothelial cell damage. It
can be used as an indicator of drug effect, both toxicity as well as efficacy. For example, in lupus
patients, drugs effectively minimizing inflammatory/coagulation mediated, large vessel injury would
result in decreasing EPCR levels.

DEPR:
The following abbreviations are used: rsEPCR, recombinant soluble EPCR with the HPC4 epitope
inserted in place of the transmembrane domain and cytosolic tail; mAb, monoclonal antibody;
SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

DEPR:
Proteins. Human protein C (Esmon, et al. 1993. Methods Enzymol. 222:359-385), bovine thrombin (Owen,
et al. 1974. J. Biol. Chem. 249:594-605), and bovine antithrombin (Esmon 1977. "Factors regulating
the inhibition of thrombin by antithrombin III. In Chemistry and Biology of Thrombin". R. L.
Lundblad, J. W. Fenton, II, and K. G. Mann, editors. Ann Arbor Science, Ann Arbor. 403-411) were
purified as described. Recombinant soluble EPCR, rsEPCR, consists of the extracellular domain of
EPCR truncated at residue 210 just before the transmembrane domain, followed by a 12 residue
sequence that permits calcium-dependent immunoaffinity purification on the HPC4 monoclonal antibody
(Takahashi, et al. 1992; Stearns, et al. 1988. J. Biol. Chem. 263:826-832). The construction,
purification, and protein C/APC binding characteristics of rsEPCR (Fukudome, et al. 1996). Goat
preimmune serum and polyclonal antiserum to rsEPCR was prepared and the IgG purified (Fukudome, et
al. 1996). Goat anti-rsEPCR polyclonal antibody was biotinylated with biotinamidocaproate
N-hydroxysuccinimide ester using standard procedures.

DEPR:
Monoclonal antibodies. Monoclonal antibodies (mAb) against rsEPCR were obtained as described for
other proteins (Esmon, et al. 1993). The 1494, 1495, and 1496 mAb are IgG1k antibodies that bind to
rsEPCR and to cell surface-expressed EPCR. The 1494 and 1496 mAb block the binding of protein C and
APC to EPCR and inhibit the ability of cellular EPCR to facilitate protein C activation by the
thrombin-thrombomodulin complex (Stearns-Kurosawa, et al. 1996). The 1495 mAb does not block ligand
binding to EPCR, does not alter cell surface protein C activation and has a binding epitope distinct
from that for 1494 or 1496 mAb. The 1494 and 1495 mAbs were biotinylated with biotinamidocaproate
N-hydroxysuccinimide ester using standard procedures. The 1494 mAb was coupled to AffiGel-10,
according to the manufacturer's directions, for immunoaffinity purification of plasma EPCR. The
screening of anti-EPCR mAb was done using methods described by Stearns-Kurosawa, et al. (1996);
Fukudome, et al. (1996).

DEPR:
Clotting Assay. The effect of rsEPCR or purified plasma EPCR on APC (25 nM) anticoagulant activity
in a one-stage factor Xa clotting assay was performed (Regan, et al. 1996) in the presence or
absence of 83 .mu.g/ml 1496 mAb, an antibody that blocks APC-EPCR interaction (Stearns-Kurosawa, et
al. 1996). The soluble EPCRs and 1496 mAb were pre-incubated for 15 minutes before assay.

DEPR:
Flow Cytometric Analysis. To serve as a fluorescent probe, APC was labeled with fluorescein in the
active site (fl-APC) as described (Fukudome and Esmon, 1994; Bock, P. E. 1988. Biochemistry
27:6633-6639). The effect of rsEPCR or plasma EPCR on APC binding to EA.hy926 cells was studied by
flow cytometry (Fukudome, et al. 1996). Briefly, harvested cells were incubated for 30 min on ice
with 60 nM fl-APC in the absence or presence of increasing concentrations of either soluble EPCR
preparation, washed, and cell-bound fluorescence was determined by flow cytometry with 10,000 events
counted per sample. All assays were done in Hank's balanced salt solution supplemented with 1%
bovine serum albumin, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, and 0.02% sodium azide.

DEPR:
Cell surface protein C activation. EA.hy926 cells were cultured in 96-well tissue culture dishes
(Stearns-Kurosawa, et al. 1996). The confluent monolayers were washed three times with Hank's
balanced salt solution supplemented with 1% (w/v) bovine serum albumin, 3 mM CaCl.sub.2, 0.6 mM
MgCl.sub.2, and 0.02% sodium azide. All assays were done at room temperature in the same buffer in
60 .mu.l final volume, and all protein concentrations represent the final concentration in the
assay. Protein C was added (0.1 .mu.M) in the absence or presence of rsEPCR, plasma EPCR, or 1494
mAb at the indicated concentrations and pre-incubated with the cells for 15 minutes. Thrombin was
added to the mixtures (2 nM) to start the activation reactions. At the indicated time, 50 .mu.l
aliquots were removed and added to 10 .mu.l of antithrombin (0.7 .mu.M final) and heparin (5 U/ml
final) in a 96-well microtiter plate. APC amidolytic activity was determined by addition of
Spectrozyme PCa substrate (0.2 mM) and the rate of change in absorbance at 405 nm (mOD/min) was
measured on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, Calif.). All assay
points were done in triplicate wells and less than 10% of the protein C substrate was activated as
determined by reference to a standard curve of fully activated protein C versus mOD/min.

DEPR:
ELISA for quantitation of plasma EPCR. An enzyme-linked immunosorbent assay for detection of EPCR
antigen in plasma was developed. Microtitre plates (Maxisorp; Nunc, Roskilde, Denmark) were coated
with 50 .mu.l of 4 .mu.g/ml 1495 mAb in 15 mM Na.sub.2 CO.sub.3, 35 mM NaHCO.sub.3, pH 9.6 at
4.degree. C. overnight. The following steps were done at room temperature. The wells were washed
three times with 20 mM Tris-HCl, 0.1M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer) and blocked with
assay buffer containing 0.1% (w/v) gelatin for at least one hour. The wells were washed, 50 .mu.l
samples were added in triplicate wells, and the plates were incubated for one hour. The wells were
aspirated, washed three times with assay buffer and 50 .mu.l of 2 .mu.g/ml biotin-1494 mAb was
added. The plates were incubated for one hour, washed three times and 50 .mu.l of 0.25 .mu.g/ml
streptavidin-alkaline phosphatase conjugate (GibcoBRL) was added and incubated for an additional one
hour. The wells were washed five times and the substrate and amplifier reagents from an ELISA
amplification kit (GibcoBRL) were added sequentially at 15 minute intervals according to the
manufacturer's directions. The color development was stopped with 0.3M H.sub.2 SO.sub.4 and the
endpoint absorbance at 490 nm was read on a Vmax microplate reader. Each plate contained standards
in triplicate wells from 1.5-100 ng/ml rsEPCR in 20 mM Tris-HCl, 0.1M NaCl, 1 mM EDTA, 0.1% gelatin,
pH 7.5. The standard curve was linear (r=0.99) from 1.5-12.5 ng/ml and plasma samples were diluted
with the same buffer to fall within the linear range. Preliminary experiments determined that a
final concentration of 1-2% human plasma did not affect the linearity or sensitivity of the standard
curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1 mM EDTA to
a final 2% plasma and EPCR L, antigen levels were calculated from the average of triplicate wells by
reference to a standard curve determined on the same plate.

DEPR:
An alternative assay was developed in which the coating and detecting antibodies were reversed (1494
mAb coating; biotin-1495 mAb detecting) and antibody binding was detected with the Blue Phos
substrate (KPL Laboratories; Gaithersburg, Md.). this method was used to assay plasma EPCR in the

sepsis patients. This assay was more sensitive, probably because of affinity differences, but both assays gave qualitatively similar results.

DEPR:
Purification of plasma EPCR. Plasma EPCR as purified from human citrated plasma (Oklahoma Blood Institute) using a combination of ion-exchange chromatography, anti-rsEPCR mAb immunoaffinity chromatography, and chromatography on protein C affinity columns. Two preparations were done in slightly different ways.

DEPR:
In the first preparation, plasma (1L) was diluted with an equal volume of 20 mM Tris-HCl, pH 7.5, 10 mM benzamidine, 400 units sodium heparin and batch-adsorbed for 1 hour with 1 g pre-swollen QAE resin. After settling, the resin was processed for purification of protein C (Esmon, et al. 1993).
Solid ammonium sulfate was added to the supernatant at 4.degree. C. to 40% saturation, centrifuged, and additional ammonium sulfate was added to that supernatant to achieve 70% saturation. After centrifugation, the soft pellet was placed in dialysis bags and dialyzed overnight against 12 L of 20 mM Tris-HCl, 0.02% sodium azide, pH 7.4. The dialysate was applied to a 1496 mAb-AffiGel-10 immunoaffinity column (6 ml resin; 5 mg IgG/ml resin) equilibrated in 20 mM Tris-HCl, 0.1M NaCl, 0.02% sodium azide, pH 7.4. The column was washed with more than 12 ml of the same buffer and eluted with 50% (v/v) ethylene glycol in 20 mM Tris-HCl, pH 7.4 (Jun Xu, unpublished observations). The peak fractions from the elution were pooled (0.37 total OD.sub.280), concentrated (Centriprep 30, Millipore), and the buffer exchanged to 20 mM Tris-HCl, 0.1M NaCl, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, 0.02% sodium azide, pH 7.4. This material was applied to a protein C affinity column that had been previously prepared by applying the purified protein C (3 mg) to an HPC4-AffiGel-10 column (5 mg IgG/ml resin; 0.9.times.8 cm) in the same buffer. The HPC4 mAb binds the protein C activation region in a calcium-dependent fashion (Esmon, et al. 1993; Stearns, et al. 1988) and does not interfere with subsequent binding of EPCR to protein C. After applying the sample containing plasma EPCR, the column was washed with approximately 12 ml of buffer and eluted with 20 mM Tris-HCl, 0.1M NaCl 5 mM EDTA, 10 mM MOPS, 0.02% sodium azide, pH 7.5. Fractions were monitored for absorbance at 280 nm and for EPCR antigen using the ELISA described above. The eluate containing both protein C and plasma EPCR was applied to an FPLC (Pharmacia-LKB, Uppsala, Sweden) Mono Q column and the column developed with a linear gradient of 0.1-1M NaCl in 20 mM Tris-HCl , pH 7.5. About half of the plasma EPCR did not bind to the Mono Q column, half eluted at about 0.2M NaCl, and the protein C eluted at approximately 0.5M NaCl. Both ionic species of plasma EPCR appeared identical on SDS-PAGE gels under reducing or non-reducing conditions with silver staining, with Coomassie BB staining or with gold staining (Pierce) after transfer to PVDF membranes, and on Western blots with the biotin-polyclonal anti-rsEPCR antibody probe.

DEPR:
The second preparation of plasma EPCR was done starting with 4L of plasma to purify enough protein for functional studies. In this case, the 1496-AffiGel-10 resin (20 ml of 5 mg IgG/ml resin) was added directly to the citrated plasma, along with final concentrations of 10 mM benzamidine, 1 mM diisopropylfluorophosphate, and 0.5 units/ml sodium heparin. The plasma was batch-adsorbed overnight at 4.degree. C. with gentle mixing. After the resin settled, the supernatant was processed for protein C purification (Esmon, et al. 1993). The resin was packed into a 2.5.times.30 cm column, washed extensively with 20 mM Tris-HCl, 0.1M NaCl, 0.02% sodium azide, pH 74 and eluted with 50% ethylene glycol in 20 mM Tris-HC1, pH 7.4. The eluate was pooled and concentrated (5.5 total OD.sub.280), applied to a Mono Q column and the two ionic species (breakthrough and 0.2M NaCl eluate peak) were re-applied to the 1496-AffiGel-10 resin (1.5.times.11 cm). The column was eluted with 50% ethylene glycol as before. The eluate (0.71 ODs) was concentrated and the buffer exchanged to 20 mM Tris-HCl, 0.1M NaCl, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, 0.02% sodium azide with a Centriprep 30.
This material was then applied to an affinity column in which protein C (2.9 mg) had been initially applied in the same buffer to an HPC2-AffiGel-10 column (0.6.times.17 cm). The HPC2 mAb binds to the protein C serine protease domain and does not interfere with EPCR binding (Fukudome, et al. 1996).
The bound EPCR was eluted with buffer containing 5 mM EDTA. Contaminating serum amyloid P (from the protein C sample) was removed by ion-exchange chromatography on the FPLC Mono Q column. The sample was applied in 0.2M NaCl, so that the plasma EPCR did not bind, and was separated from the contaminants which eluted at 0.4-0.5M NaCl. The resultant purified plasma EPCR (0.193 OD.sub.280) appeared homogenous by SDS-PAGE with silver staining and by Western blotting with polyclonal anti-rsEPCR. This material was used for the functional studies and amino-terminal sequence analysis.

DEPR:
Protein Sequencing. The amino-terminal sequence analysis of soluble plasma EPCR was performed in Dr. Kenneth Jackson's laboratory at the Molecular Biology Research Facility, W. K. Warren Medical Research Institute, Oklahoma City. Amino acids are designated by the standard one letter code.

DEPR:
Plasma EPCR purity was determined from silver stained SDS-PAGE 10% gels and western blots of membranes probed with biotin-goat anti-rsEPCR (reduced and non-reduced). A single band of approximately 43,000 Da appears in both the serum and plasma samples after the membrane is probed with the polyclonal antibody. The size of the protein detected appears slightly larger than the rsEPCR. The other bands detected were background binding of IgG as judged by probing with preimmune IgG and longer exposure times. overnight incubation of plasma samples with the anti-EPCR 1495 mAb coupled to AffiGel-10 resin, followed by washing and elution of bound antigen under reducing conditions, resulted in a single band detected by Western blotting with biotin-goat anti-rsEPCR polyclonal antibody.

DEPR:
Determination of soluble EPCR antigen in plasma from healthy volunteers by ELISA using mAb 1495 as the coating antibody found antigen levels of 91.1 +/- 24.5 ng/ml in females (n=12) and 107.2 +/- 30.2 ng/ml in males (n=10). When calculated together, the average plasma EPCR antigen level was 98.4 +/- 27.8 ng/ml. The value for males appeared to be slightly higher than for females, similar to thrombomodulin (Quehenberger, et al. Thromb. Haemost. 76: 729-734), although the population studied was too limited for statistical analysis and this study was not designed to assess differences due to gender, age, diet or other variables.

DEPR:

Since the plasma EPCR appeared to be a single species at approximately 100 ng/ml, it became important to determine whether the circulating EPCR could bind protein C and APC. Soluble EPCR was purified from human plasma by a combination of ion-exchange chromatography, precipitation with ammonium sulfate, and immunoadsorption by anti-EPCR 1496 mAb-AffiGel-10 column chromatography as described in Experimental Procedures.

DEPR:
This plasma EPCR (approximately 110 .mu.g) was applied to a protein C affinity column prepared by applying protein C (3 mg) to an anti-protein C HPC4 mAb-AffiGel-10 column in buffer containing 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2 The column was washed and plasma EPCR was applied at fraction 19. The column was washed and eluted with buffer containing 5 mM EDTA starting at fraction 35. Absorbance at 280 nm and EPCR antigen was determined for the fractions. EPCR antigen was determined by ELISA.

DEPR:
More than 98% of the applied plasma EPCR antigen bound to the protein C affinity column. The absorbance profile indicates co-elution of EPCR and protein C from the antibody column, consistent with the calcium-dependence of protein C binding to this antibody (Stearns, et al. 1988).

DEPR:
To purify sufficient protein for functional and structural studies, EPCR was purified from 4L of plasma using a similar, but slightly modified procedure. After elution from a protein C-antibody affinity column, residual contaminating proteins were removed by ion-exchange chromatography on an FPLC Mono Q column. The resultant preparation of plasma EPCR appeared homogenous on SDS-PAGE 10% gels with silver staining and identical results were obtained with western blots probed with biotin-goat anti-rsEPCR polyclonal antibody under both reducing and non-reducing conditions.
Amino-terminal sequence analysis of the purified protein yielded only one sequence, S-Q-D-A-S-D, which is identical to the amino-terminal sequence of recombinant soluble EPCR (Sequence ID No. 2).
This is the first amino-terminal sequence determination of EPCR from a natural source.

DEPR:
The ability of plasma EPCR to bind to APC was assessed by competition studies in which plasma EPCR was allowed to compete with cellular EPCR for APC, and the resultant free APC that could bind to cellular EPCR was assessed by flow cytometry (FIG. 4a). APC labeled with fluorescein in the active site (fl-APC) was incubated with EA.hy926 cells in the presence or absence of either plasma EPCR or rsEPCR. The EPCR concentration dependence for inhibition of APC binding to the cells was similar for both soluble forms of EPCR. This observation indicates that the affinity of plasma EPCR for binding APC is similar to that previously determined for the rsEPCR-APC binding interaction (Kd.sub.app approximately 30 nM).

DEPR:
While rsEPCR has little effect on protein C activation in a soluble system (Regan, et al. 1996), membrane-bound EPCR has a very potent ability to facilitate activation on cell surfaces (Stearns-Kurosawa, et al. 1996). The current data demonstrating the existence of a circulating form of EPCR capable of binding protein C and APC suggested that plasma EPCR has the potential to alter cell-surface activation of protein C. The thrombin-dependent activation of an approximately physiological level of protein C (0.1 .mu.M) on EA.hy926 cells was inhibited by excess rsEPCR almost to the level of that observed with the anti-rsEPCR 1494 mAb that blocks the EPCR-protein C binding interaction, as shown by FIG. 4b. Previous studies have demonstrated that rsEPCR has no effect on APC amidolytic activity using small synthetic substrates (Regan, et al. 1996). The plasma EPCR was slightly more effective in its ability to inhibit cell-surface protein C activation on the EA.hy926 cells relative to the rsEPCR, as shown by FIG. 4c.

DEPR:
In a one-stage factor Xa clotting assay, purified plasma and soluble recombinant EPCR inhibited the APC prolongation of clotting times similarly (FIG. 4d). Inhibition of APC anticoagulant activity by rsEPCR had been observed previously (Regan, et al. 1996). As expected, the 1496 mAb reversed this effect by blocking the APC-plasma EPCR binding interaction.

DEPR:
To address the question of whether soluble EPCR is present in urine, four urine samples were collected (first morning void) and analyzed for the presence of soluble EPCR by western blotting and ELISA.

DEPR:
Undiluted pediatric urine samples were compared to a 4% normal plasma and recombinant soluble EPCR (1 ng). The samples were incubated with biotin-goat-anti-rsEPCR and a streptavidin-alkaline phosphatase detection system.

DEPR:
The western blot indicates that a) soluble EPCR is present in urine, and b) the soluble EPCR antigen is present at a size similar to that observed in plasma. Obvious degradation is not observed. The amount of soluble EPCR in the four samples as quantified by ELISA was 40.3, 6.1, 35.6, and 90.1 ng/ml.

DEPR:
Normal human plasma EPCR concentration are about 100 ng/ml (98.4.+-.27.8 ng/ml; 2.5 nM), as discussed above. A panel of samples from patients with lupus erythematosus (n=54) was assayed and soluble EPCR levels were found to range from non-detectable levels to greater than 1,700 ng/ml.
Fifteen patients had soluble EPCR levels greater than 200 ng/ml.

DEPR:
Previous studies have shown elevated soluble plasma TM levels in lupus patients due to endothelial damage and the current lupus patient samples were assayed for plasma TM as a reference. It was found that their soluble TM levels had absolutely no correlation with their soluble EPCR levels, as shown by FIG. 5. This is an important observation that suggests that the source of the soluble plasma EPCR is not simply from randomly damaged endothelium. In contrast to TM, membrane-bound EPCR expression in humans and primates is restricted primarily to the endothelium of large vessels, with capillaries expressing little EPCR. The distinctive localization of EPCR is expected to augment protein C activation locally to prevent large vessel thrombosis. The primary localization of membrane-bound EPCR to the large vessels points to a targeted thrombotic risk in the large vessels that may be predicted by soluble plasma EPCR concentrations.

DEPR:
Patient blood samples were taken at time 0 (entry into the Intensive Care Unit, ICU) and at two days and six days after treatment with anti-thrombin II (ATIII) or a placebo.

Plasma soluble EPCR and
soluble thrombomodulin (TM) were assayed only on time 0 samples.

DEPR:
These results are shown graphically in FIG. 6. As in the lupus patients, patients with sepsis show
very significant elevations in plasma EPCR levels, not correlated with soluble TM levels.

DEPR:
The observation that soluble plasma EPCR inhibits both protein C activation and activated protein C
anticoagulant activity indicates that the elevated plasma EPCR levels in these patients poses an
additional thrombotic risk and marks evidence of vascular injury/responsiveness. Examples of
conditions these are indicative of include disorders associated with endothelial cell stimulation,
atherogenesis, leukocyte adhesion and plaque rupture.

DEPR:
As an initial approach to determine whether a soluble EPCR isoform could be generated by alternative
splicing mechanism, RNA was isolated from human and baboon tissues and reverse transcriptase-PCR
(RT-PCR) performed with gene-specific primers. Although the baboon EPCR genomic sequence is not
known, primers based on the human sequence were used based on the reasoning that baboons and humans
are closely related on the evolutionary scale.

DEPR:
In the RT-PCR procedure generally, total RNA is isolated from homogenized tissue. The RNA is mixed
with a specific antisense primer, nucleotides and the reverse transcriptase enzyme. In the mix, the
RNA serves as a template for the reverse transcriptase to create a first strand cDNA. This new cDNA
template is then amplified by conventional PCR using specific primers and Taq polymerase. Primers
that would amplify both the membrane form of EPCR (424 bp) and the predicted alternatively spliced
product (674 bp) were chosen. Products corresponding to both forms of EPCR were amplified from a
variety of baboon tissues (FIG. 4) and human lung and placenta. Possible contamination with genomic
DNA was unlikely as judged by controls without reverse transcriptase and the lack of a 1,885 bp band
in the reactions with the tissues.

DEPR:
A. EPCR ELISA: The coating antibody is 1494 mAb that binds to the ligand binding domain of EPCR. The
detecting antibody is biotinylated 1495 mAb, which does not block protein C/APC binding, and does
not cross-react with 1494 mAb. The detection system is streptavidin-alkaline phosphatase and
BluePhos substrate (from KPL).

DEPR:
The observation that the predicted soluble EPCR isoforms will have unique carboxyl terminal tails
provides a structural difference for distinguishing between the isoforms using isoform-specific
antibodies. The working model is that plasma levels of proteolyzed soluble EPCR will report
endothelial injury, whereas levels of alternatively spliced soluble EPCR will report an endothelial
response to stimuli. It is anticipated that the relative plasma levels of the soluble EPCR isoforms
will provide information on large vessel endothelial dysfunction and injury in specific pathologies.

DEPR:
RT-PCR products from human tissues: placenta, lung, and tongue, were electrophoresed using the
CRES/CREA primers specific for EPCR. The procedures were the same as used for the baboon tissues.
Products corresponding to the membrane isoform of EPCR (mEPCR) and the alternatively-spliced soluble
EPCR isoform (sEPCR) were observed. The products look essentially the same as that seen using the
baboon tissues. The only difference is that the placental tissue appears to have additional
products.

DEPC:
Detection and Characterization of Soluble EPCR; Physiological Role and Utility as a Marker

DEPC:
Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPC:
Identification of Functional Endothelial Protein C Receptor in Human Plasma

DEPC:
Detection of Soluble EPCR in Urine.

DEPC:
Measurement of Plasma EPCR from Lupus Patients.

DEPC:
Plasma Soluble EPCR in Septic Shock Patients.

DEPC:
Identification of Alternatively Spliced forms of EPCR in Baboon and Human Tissues.

DETL:
TABLE 1 _____ Plasma soluble receptor levels Plasma EPCR Plasma TM
ng/ml ng/ml _____ Normal volunteers, 133.4 .+-. 53.4 35.5 .+-. 20.4
n = 20 Systemic lupus 262.1 .+-. 154.5* 104.7 .+-. 77.5* erythematosus, (P = 0.0004) (P = 0.0008) n
= 40 Sepsis, n = 24 224.9 .+-. 74.5* 39.9 .+-. 73.1 (P = 0.00009)
_____ *Significant difference between the means relative to normal;
unpaired Student's t test.

DETL:

SEQUENCE LISTING (1)
GENERAL INFORMATION: (iii) NUMBER OF SEQUENCES: 8 (2) INFORMATION FOR SEQ ID NO:1: (i) SEQUENCE
CHARACTERISTICS: (A) LENGTH: 1302 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D)
TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE:
(A) NAME/KEY: misc.sub.-- feature (B) LOCATION: 1..1302 (D) OTHER INFORMATION: /note= "Nucleotides
25 through 738 encode the Endothelial Cell Protein Receptor of Sequence ID No. 2." (xi) SEQUENCE
DESCRIPTION: SEQ ID NO:1:
CAGGTCCGGAGCCTCAACTTCAGGATGTTGACAACATTGCTGCCG
ATACTGCTGCTGTCT60

GGCTGGGCCTTTTGTAGCCAAGACGCCTCAGATGGCCTCCAAAGA
CTTCATATGCTCCAG120

ATCTCCTACTTCCGCGACCCCTATCACGTGTGGTACCAGGGCAAC
GCGTCGCTGGGGGGA180

CACCTAACGCACGTGCTGGAAGGCCCAGACACCAACACCACGATC
ATTCAGCTGCAGCCC240

TTGCAGGAGCCCGAGAGCTGGGCGCGCACGCAGAGTGGCCTGCA
GTCCTACCTGCTCCAG300

TTCCACGGCCTCGTGCGCCTGGTGCACCAGGAGCGGACCTTGGCC
TTTCCTCTGACCATC360

CGCTGCTTCCTGGGCTGTGAGCTGCCTCCCGAGGGCTCTAGAGCC
CATGTCTTCTTCGAA420

GTGGCTGTGAATGGGAGCTCCTTTGTGAGTTTCCGGCCGGAGAGA
GCCTTGTGGCAGGCA480

GACACCCAGGTCACCTCCGGAGTGGTCACCTTCACCCTGCAGCAG
CTCAATGCCTACAAC540

CGCACTCGGTATGAACTGCGGGAATTCCTGGAGGACACCTGTGTG
CAGTATGTGCAGAAA600

CATATTTCCGCGGAAAACACGAAAGGGAGCCAAACAAGCCGCTCC
TACACTTCGCTGGTC660

CTGGGCGTCCTGGTGGGCGGTTTCATCATTGCTGGTGTGGCTGTAG
GCATCTTCCTGTGC720

ACAGGTGGACGGCGATGTTAATTACTCTCCAGCCCCGTCAGAAGG
GGCTGGATTGATGGA780

GGCTGGCAAGGGAAAGTTTCAGCTCACTGTGAAGCCAGACTCCCC
AACTGAAACACCAGA840

AGGTTTGGAGTGACAGCTCCTTTCTTCTCCCACATCTGCCCACTGA
AGATTTGAGGGAGG900

GGAGATGGAGAGGAGAGGTGGACAAAGTACTTGGTTTGCTAAGA
ACCTAAGAACGTGTAT960

GCTTTGCTGAATTAGTCTGATAAGTGAATGTTTATCTATCTTTGTG
GAAAACAGATAATG1020

GAGTTGGGGCAGGAAGCCTATGCGCCATCCTCCAAAGACAGACAG
AATCACCTGAGGCGT1080

TCAAAAGATATAACCAAATAAACAAGTCATCCACAATCAAAATAC
AACATTCAATACTTC1140

CAGGTGTGTCAGACTTGGGATGGGACGCTGATATAATAGGGTAGA
AAGAAGTAACACGAA1200

GAAGTGGTGGAAATGTAAAATCCAAGTCATATGGCAGTGATCAAT
TATTAATCAATTAAT1260

AATATTAATAAATTTCTTATATTTAAAAAAAAAAAAAAAAAA1302
(2) INFORMATION FOR SEQ ID NO:2: (i) SEQUENCE
CHARACTERISTICS: (A) LENGTH: 238 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE
TYPE: protein (iii) HYPOTHETICAL: NO (ix) FEATURE: (A) NAME/KEY: misc.sub.-- feature (B) LOCATION:
1..365 (D) OTHER INFORMATION: /note= "Endothelial Cell Protein Receptor encoded by nucleotides 1
through 1302 of Sequence ID No. 1." (ix) FEATURE: (A) NAME/KEY: Modified-site (B) LOCATION: 1..15
(D) OTHER INFORMATION: /note= "Amino acids 1-15 represent a putative signal sequence." (ix) FEATURE:
(A) NAME/KEY: Domain (B) LOCATION: 211..236 (D) OTHER INFORMATION: /note= "Amino acids 211-236
represent a putative transmembrane domain." (ix) FEATURE: (A) NAME/KEY: Active-site (B) LOCATION:
47..174 (D) OTHER INFORMATION: /note= "Amino acids 47-49, 64-66, 136-138 and 172-174 represent
potential N- glycosylation sites." (ix) FEATURE: (A) NAME/KEY: Active-site (B) LOCATION: Cys 17 (D)
OTHER INFORMATION: /note=immediately preceeds amino acid cleavage site (ix) FEATURE: (A) NAME/KEY:
Active-site (B) LOCATION: Gly 201 (D) OTHER INFORMATION: /note=peptide inserts in alternatively
spliced EPCR (ix) FEATURE: (A) NAME/KEY: Modified-site (B) LOCATION: 17..186 (D) OTHER INFORMATION:
/note= "Amino acids 17, 114, 118 and 186 represent extracellular cysteine residues." (xi) SEQUENCE
DESCRIPTION: SEQ ID NO:2:
MetLeuThrThrLeuLeuProIleLeuLeuLeuSerGlyTrpAlaPhe 151015
CysSerGlnAspAlaSerAspGlyLeuGlnArgLeuHisMetLeuGln 202530
IleSerTyrPheArgAspProTyrHisValTrpTyrGlnGlyAsnAla 354045
SerLeuGlyGlyHisLeuThrHisValLeuGluGlyProAspThrAsn 505560
ThrThrIleIleGlnLeuGlnProLeuGlnGluProGluSerTrpAla 65707580
ArgThrGlnSerGlyLeuGlnSerTyrLeuLeuGlnPheHisGlyLeu 859095
ValArgLeuValHisGlnGluArgThrLeuAlaPheProLeuThrIle 100105110
ArgCysPheLeuGlyCysGluLeuProProGluGlySerArgAlaHis 115120125
ValPhePheGluValAlaValAsnGlySerSerPheValSerPheArg 130135140
ProGluArgAlaLeuTrpGlnAlaAspThrGlnValThrSerGlyVal 145150155160
ValThrPheThrLeuGlnGlnLeuAsnAlaTyrAsnArgThrArgTyr 165170175
GluLeuArgGluPheLeuGluAspThrCysValGlnTyrValGlnLys 180185190
HisIleSerAlaGluAsnThrLysGlySerGlnThrSerArgSerTyr 195200205
ThrSerLeuValLeuGlyValLeuValGlyGlyPheIleIleAlaGly 210215220
ValAlaValGlyIlePheLeuCysThrGlyGlyArgArgCys 225230235 (2)
INFORMATION FOR SEQ ID NO:3: (i) SEQUENCE
CHARACTERISTICS: (A) LENGTH: 48 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE
TYPE: protein (iii) HYPOTHETICAL: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
MetMetGlyArgGlyProGlyLysGlnAlaGlyGluArgAlaGlySer 151015
ArgGlnMetAspGlyProGluGlyTrpMetProArgAlaThrArgGly 202530
ProGlnLysGlyValTrpAspArgThrHisAlaAlaSerValSerTrp 354045 (2)
INFORMATION FOR SEQ ID NO:4: (i)
SEQUENCE CHARACTERISTICS: (A) LENGTH: 148 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single
(D) TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi)
SEQUENCE DESCRIPTION: SEQ ID NO:4:
ATGATGGGACGGGGCCCAGGCCTGCAAGCTGGGGAGAGGGCGGG
TTCCAGACAAATGGAT60

GGACCTGAAGGATGGATGCCTAGAGCAACAAGAGGCCCACAGCT
GGGGGTTTGGGACAGA120
ACACACGCAGCTTCAGTCAGTTGGTAAA148 (2)
INFORMATION FOR SEQ ID NO:5: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE:
nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii)
HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE: (A) NAME/KEY: misc.sub.-- feature (B) LOCATION:
1..24 (D) OTHER INFORMATION: /note= "5'sense primer near end of exon II." (xi) SEQUENCE DESCRIPTION:
SEQ ID NO:5: TCGTGCGCCTGGTGCACCAGGAGC24 (2)
INFORMATION FOR SEQ ID NO:6: (i) SEQUENCE
CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D)
TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE:
(A) NAME/KEY: misc.sub.-- feature (B) LOCATION: 1..24 (D) OTHER INFORMATION: /note= "3'antisense
primer within exon IV." (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
CGCCGTCCACCTGTGCACAGGAAG24 (2)
INFORMATION FOR SEQ ID NO:7: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 base pairs (B) TYPE:
nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii)
HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE: (A) NAME/KEY: misc.sub.-- feature (B) LOCATION:
1..22 (D) OTHER INFORMATION: /note= "5'sense primer near end of exon III." (xi) SEQUENCE
DESCRIPTION: SEQ ID NO:7: AGCAGCTCAATGCCTACAACCG22
(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE
CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D)
TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE:
(A) NAME/KEY: misc.sub.-- feature (B) LOCATION: 1..29 (D) OTHER INFORMATION: /note= "3'antisense
primer within exon IV." (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
CCGTAGAAGGACACGTGTCCACCTGCCGC29

CLPR:
1. An assay for soluble endothelial protein C receptor comprising

CLPR:
3. The assay of claim 1 further comprising the step of correlating the amount of soluble endothelial
protein C receptor with calibration standards.

CLPR:
8. A kit for detection and measurement of endothelial protein C receptor comprising

CLPR:
9. The kit of claim 8 where the antibody has a higher affinity for endothelial protein C receptor including the transmembrane domain than for endothelial protein C receptor not including the transmembrane domain.

CLPR:
10. The kit of claim 8 wherein the antibody is immunoreactive with the insert in an alternatively spliced endothelial protein C receptor.

CLPR:
11. The kit of claim 8 wherein the antibodies block binding of endothelial protein C receptor and activated protein C or protein C.

CLPV:
measuring the amount of soluble endothelial protein C receptor.

CLPV:
an antibody immunoreactive with endothelial protein C receptor,

CLPV:
reagents to detect a reaction between the antibody and endothelial protein C receptor in a sample from a patient, and

CLPV:
standards to correlate the amount of reaction to normal and abnormal levels of endothelial protein C receptor.

ORPL:
Fukudome and Esmon, "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein C/Activated Protein C Receptor (EPCR)--The Structural and Functional Conservation in Human, Bovine and Murine EPCR*," J. Biol. Chem. 270(10):5571-5577 (1995).

9. Document ID: US 5695993 A

L1: Entry 9 of 24 File: USPT Dec 9, 1997

US-PAT-NO: 5695993
DOCUMENT-IDENTIFIER: US 5695993 A

TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

DATE-ISSUED: December 9, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Fukudome; Kenji	Oklahoma City	OK	N/A	N/A
Esmon; Charles T.	Oklahoma City	OK	N/A	N/A

US-CL-CURRENT: 435/325; 435/320.1, 435/69.1, 536/23.5

AB: Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca.sup.2+ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 9 of 24 File: USPT Dec 9, 1997

DOCUMENT-IDENTIFIER: US 5695993 A
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ABPL:
Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca.sup.2+ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

BSPR:
An endothelial cell protein C binding protein (referred to herein as "EPCR") has been cloned and characterized. The protein is predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein C and activated protein C (Kd=30 nM) and is calcium dependent. The message and binding function of the receptor are both down regulated by cytokines such as TNF.

BSPR:
These results identify a new member of a complex pathway that, like other members of the pathway, is subject to regulation by inflammatory cytokines, and can therefore be used to modulate inflammatory reactions in which protein C or activated protein C is involved. Inhibition of the inflammatory response can be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in

contact with blood will render the surfaces anticoagulant by virtue of the ability of EPCR to bind
and concentrate the anticoagulant activated protein C at the surface. Alternatively, the function of
EPCR can be enhanced by overexpressing the EPCR in endothelium that could be used to coat vascular
grafts in patients with vascular disease or on stents in cardiac patients.

DRPR:
FIGS. 3A and 3B are flow cytometric analyses of F1-APC binding to 293T cells transfected with a cDNA
clone of EPCR. Cells were transfected with a clone EPCR/pEF-BOS or pEF-BOS (negative control) by the
calcium/phosphate method. After 24 h, cells were harvested and F1-APC binding was performed in the
absence (dotted lines) or presence of 1.3 mM CaCl2 (solid lines).

DRPR:
FIG. 4 is the predicted protein structure of EPCR based on nucleotide sequence Sequence ID No. 1,
predicted amino acid sequence, Sequence ID No. 2, and a hydropathy plot of EPCR. The signal sequence
and transmembrane region are indicated with the solid bars.

DRPR:
FIG. 5 is a comparison of the amino acid sequence of EPCR to the amino acid sequences of other
members of the CD1 family and CCD41. The EPCR sequence, Sequence ID No. 2, is shown in the first
line and compared to murine CCD41, Sequence ID No. 3, (second line), human CD1d, Sequence ID No. 4,
(third line) and murine CD1.2, Sequence ID No. 5, (fourth line). Identities with EPCR are indicated
by open boxes. Residues that are conserved between EPCR and all of the human CD1 family members are
indicated by a double asterisk. Residues shared with one or more members of the CD1 family are
indicated by a single asterisk.

DRPR:
FIG. 6 is a comparison of the amino acid sequence of human EPCR, Sequence ID No. 2, (first line) to
the amino acid sequence of murine EPCR, Sequence ID No. 6, (second line). Identities are indicated
by boxes. Similarities are indicated with an asterisk.

DEPR:
Human protein C and activated protein C are shown to bind to endothelium specifically, selectively
and saturably (Kd=30 nM, 7000 sites per cell) in a Ca.sup.2+ dependent fashion. FL-APC binding to
various human cell lines were examined, and found that the binding was HUVEC specific. A human
kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these
binding sites. A HUVEC cDNA library was constructed using the powerful mammalian expression vector,
pEF-BOS (Mizushima and Nagata, (1990) Nucleic Acids Res. 18, 5322). Plasmid DNA was prepared from
subpools of independent colonies (2,500 colonies per pool), and transfected into 293T cells, using
the method of Kaisho et al., (1994) Proc. Natl. Acad. Sci. (USA) 91, 5325. FL-APC binding was
analyzed on a flow cytometer. One of eight subpools gave a positive signal. This subpool was divided
into 20 subpools and rescreened. After three rounds of enrichment, one positive clone, EPCR-1, was
isolated. EPCR-1 carries a 1.3 kb insert. When transfected into 293T cells, this clone was capable
of expressing the calcium-dependent binding site for FL-APC on the 293T cell surface.

DEPR:
Expression cloning revealed a 1.3 kb cDNA that coded for a type I transmembrane glycoprotein capable
of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like
thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C
receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF.
Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of
this receptor for protein C in regulating the inflammatory response.

DEPR:
Total RNAs (15 .mu.g) from various cells were isolated, electrophoresed through formaldehyde agarose
gels and transferred to a nylon membrane (Hybond-N.TM., Amersham). The 483 bp Xba I fragment from
the 5' end of the EPCR cDNA was labeled by random priming according to the manufacturer's
instructions (Multiprime.TM. DNA labeling system, Amersham) and used for hybridization.

DEPR:
Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPR:
The cDNA is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which includes a
15 amino acid signal sequence (yon Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at the
N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Sequence ID No. 2
is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2
(MLTTLLPILLLSGWA) are the putative signal sequence determined by the method of yon Heijne (yon
Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVLGVLVGGFIIAGVAVGIFLCTGGR) are the
putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49,
64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at
amino acids 17, 114, 118, and 186 of Sequence ID No. 2. A potential transmembrane region (Engelman
et al., (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 321-53) consisting of 23 amino acids was
identified at the C-terminal end (beginning at amino acid 216 of Sequence ID No. 2).

DEPR:
DNA and protein database searches revealed that the sequence is related to the
centrosome-associated, cell cycle dependent murine protein, CCD41, also referred as centrocyclin
(Rothbarth et al., (1993) J. Cell Sci. 104, 19-30), as shown by FIG. 5. The similarity in the
published sequence of murine CCD41 with human EPCR led to the cloning and sequencing of the murine
EPCR. The sequence of murine EPCR is shown in FIG. 6. It is distinct from the published sequence of
CCD41.

DEPR:
The EPCR amino acid sequence was also related to, but quite distinct from, the CD1/MHC superfamily
and the murine CD1.2, as also shown by FIG. 5. Based on the homology to CD1/MHC, it is likely that
EPCR contains two domains consisting of residues 17-114 and 118-188. Of the CD1 family members, CD1d
is the most similar to EPCR. In the mouse, CCD41 is associated exclusively with the centrosome
during G.sub.1 but becomes detectable elsewhere during the cell cycle, reaching a maximum during
G.sub.2, except during the G.sub.2 /M phase (Rothbarth et al., 1993). EPCR expression appears
restricted to endothelium, which would not be expected for a cell cycle associated protein.

DEPR:
The identification of the protein C receptor on endothelium suggests that the endothelial cell binds
protein C/APC through three distinct mechanisms. In addition to EPCR, protein S can bind APC/protein

C on negatively charged membrane surfaces that include the endothelium (Stern et al., (1986) J.
Biol. Chem. 261, 713-718), but this is not cell type specific (Dahlback et al., 1992).
Thrombomodulin in complex with thrombin can bind protein C and APC (Hogg et al., 1992). On
endothelium, the protein S binding sites (Nawroth and Stern, (1986) J. Exp. Med. 163, 740-745),
thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that
inflammation can impair protein C pathway function at multiple levels.

DEPR:
The homology to the CD1/MHC family of proteins is especially interesting since it provides
indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed
.alpha.1,2 and 3. The extracellular domain of EPCR contains four Cys residues that appear to
correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two
domains have significant homology to the .alpha.1 and .alpha.2 domains of the CD1 protein family and
the .alpha.2 domain of the MHC class 1 protein, suggesting that these proteins evolved from a common
ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites
and is rich in .beta. strand structure, suggesting that it may form a .beta. sheet. Despite the
.beta. strand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6,
381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR,
residues 118-188, contains two additional N glycosylation sites and, like the CD1/MHC family, this
domain is predicted to have limited .beta. structure.

DEPR:
In vitro studies have suggested anti-inflammatory activities for APC. For instance, an unusual
carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to selectins (Grinnell at
al., (1994) Glycobiology, 4, 221-226) Modest inhibitory effects of APC have been reported on TNF
production (Grey et al., (1993) Transplant. Proc. 25, 2913-2914). EPCR could contribute to these
anti-inflammatory mechanisms. Since the homologous protein family, CD1, can be linked to CD8
(Ledbetter et al., (1985) J. Immunol. 134, 4250-4254), it is also possible that the proteins C
receptor is linked to another protein and signal through this second protein. One of the CD1 family
members, CD1d, has been reported to promote T cell responses, possibly involving binding to CD8
(Panja et al., (1993) J. Exp. Med. 178, 1115-1119). CD1b has recently been reported to serve as an
antigen presenting molecule (Porcelli et al., (1992) Nature 360, 593-597). The ability to bind
protein C/APC could then be linked either directly or indirectly to signalling via direct
interaction with cells of the immune system. Since the MHC class of proteins is involved in
presentation of proteins to cell receptors, the concept of presentation of protein C/APC to
inflammatory cells as a means of elaborating anti-inflammatory activity may also be involved. This
includes modulation of enzyme specificity such as occurs with thrombin-thrombomodulin interaction
(Esmon, 1989). In this case, the EPCR-APC complex might cleave biologically active peptides from
unknown substrates.

DEPR:
EPCR mRNA Levels and APC Binding

DEPR:
To determine the cellular specificity of EPCR expression, the intensity of FL-APC binding to HUVEC
was compared to several human cell lines. F1-APC bound strongly only to HUVEC, and not to any of the
T, B, or monocytic cell lines tested. Cells were incubated at room temperature without or with 160
nM F1-APC in the presence of 1.3 mM CaCl.sub.2. Binding was analyzed by flow cytometry. Slight
binding was demonstrated with the osteosarcoma line, HOS and the epidermoid carcinoma cell line,
HEp-2.

DEPR:
Total RNA was extracted from these cells and hybridized with the EPCR cDNA probe for Northern Blot
Analysis. EPCR mRNA was detected by Northern blot analysis for HUVEC, Jurkat, HEp-2, Raji, HOS, and
U937. Among the cells lines tested, EPCR mRNA was detected at high levels only in HUVEC. The
calculated mRNA size of 1.3 kb was identical to the size of the isolated cDNA. After prolonged
exposure, a weak signal was also detected with the osteosarcoma cell line HOS and monocyte cell line
U937. Thus, both APC binding and EPCR mRNA expression are very specific for endothelium.

DEPR:
Effects of TNF on APC Binding and EPCR MRNA Levels

DEPR:
Several other members of the protein C anticoagulant pathway are subject to regulation by
inflammatory cytokines (Esmon, 1989). For instance, endothelial cell surface thrombomodulin
expression and message are known to be reduced by exposure of the cells to TNF (Conway and
Rosenberg, 1988; Lentz et al., 1991). To determine if a similar process occurs with EPCR, HUVEC were
treated with TNF and APC binding and expression of EPCR mRNA were examined. APC binding to HUVEC
decreased in a time dependent fashion. EPCR activity decreased more rapidly than thrombomodulin
antigen. HUVEC were cultured for 0, 6, 24 and 48 hr, in the presence of TNF-.alpha. (10 ng/ml).
Cells were harvested and residual F1-APC binding or thrombomodulin (TM) expression was analyzed by
flow cytometry. Cell surface TM was stained with an anti-TM murine monoclonal antibody and
FITC-conjugated anti-mouse IgG. The negative control is without added fluorescent ligand.

DEPR:
HUVEC were treated with 10 ng/ml of TNF-.alpha. for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message
was extracted and detected as described above. The results demonstrated that the concentration of
EPCR mRNA was also reduced by TNF treatment. Message levels and APC binding activity decreased in
parallel. Therefore, the TNF mediated down-regulation of APC binding to endothelium probably occurs
at the level of mRNA expression.

DEPR:
Enhancement of inflammatory responses by blocking binding of endogenous molecules to ECPCR can be
achieved by administration of compounds binding to the receptor to a subject in need of inhibition.
The degree of binding is routinely determined using assays such as those described above. Compounds
which are effective include antibodies to the protein, fragments of antibodies retaining the binding
regions, and peptide fragments of APC which include the Gla region. Inhibition of the inflammatory
response could be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in
contact with blood would render the surfaces anticoagulant by virtue of the ability of EPCR to bind
and concentrate the anticoagulant APC at the surface. Alternatively, the function of EPCR could be
enhanced by overexpressing the EPCR in endothelium used to coat

vascular grafts in patients with
vascular disease or on stents in cardiac patients.

DEPR:
Patients with thrombosis or hyperinflammatory conditions could be screened for defects in the EPCR
gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more
preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful
in this screening using hybridization assays of patient samples, including blood and tissues.
Screening can also be accomplished using antibodies, typically labelled with a fluorescent,
radiolabelled, or enzymatic label, or by isolation of target cells and screening for binding
activity, as described in the examples above. Typically, one would screen for expression on either a
qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with
.sup.32 P, .sup.35 S, fluorescein, biotin, or other labels routinely used with methods known to
those skilled in the art for labelling of proteins and/or nucleic acid sequences.

DEPR:
In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be
advantageous to increase its concentration in vivo on endothelium. The binding assays described here
and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken
with thrombomodulin and investigators have shown that cyclic AMP (Maruyama, I. et al. (1991)
Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis,S. et al., (1991) Blood 78, 410-415)
can elevate thrombomodulin expression. The ability to screen such drugs or drugs that block TNF down
regulation of EPCR provide an approach to elevating EPCR expression in vivo and thus enhancing
anticoagulant and anti-inflammatory activity.

DEPR:
Studies based on inhibition of binding are predictive for indirect effects of alteration of receptor
binding. For example, inhibition of binding of APC or increased expression of TNF is predictive of
inhibition of EPCR function.

DEPR:
As described herein, a variety of compounds can be used to inhibit or enhance expression of the
EPCR. The nature of the disorder will determine if the expression should be enhanced or inhibited.
For example, based on the studies involving the use of an anti-protein C antibody in combination
with cytokine, it should be possible to treat solid tumors by enhancing an inflammatory response
involving blocking of protein C or activated protein C binding to an endothelial cell protein
C/activated protein C receptor by administering to a patient in need of treatment thereof an amount
of a compound blocking binding of protein C or activated protein C to the receptor. Similarly, it
should be possible to treat disorders such as gram negative sepsis, stroke, thrombosis, septic
shock, adult respiratory distress syndrome, and pulmonary emboli using a method for inhibiting an
inflammatory response involving administration of EPCR or EPCR fragments or substances that
upregulate EPCR expression to a patient in need of treatment therof.

DEPL:
I. Cloning and Characterization of EPCR

DEPL:
II. Modulation of Inflammation using EPCR.

ORPL:
Fukudome, K. et al., "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein
C/activated Protein C Receptor (EPCR)", J. Biological Chemistry, vol. 270, no. 10, pp. 5571-5577
(1995).

10. Document ID: US 5548796 A

L1: Entry 10 of 24 File: USPT Aug 20, 1996

US-PAT-NO: 5548796
DOCUMENT-IDENTIFIER: US 5548796 A

TITLE: Method of automatic retransmission of frames in a local area network

DATE-ISSUED: August 20, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Ketchum; Kevin D.	Folsom	CA	N/A	N/A

US-CL-CURRENT: 710/52; 370/447

AB: A configurable network interface controller that provides for the automatic
retransmission of collided Ethernet frames from a local RAM while observing two modes of
operation: (1) retransmission of as much of the frame as possible without violating latency
requirements and (2) first guaranteeing the safe retransmission of the first 64 bytes and then
returning to observation of the latency requirements.

L1: Entry 10 of 24 File: USPT Aug 20, 1996

DOCUMENT-IDENTIFIER: US 5548796 A
TITLE: Method of automatic retransmission of frames in a local area network

DRPR:
FIG. 38 is a table that describes the EEPROM Configuration Register (EPCR) of the SCENIC device.

DEPC:
EEPROM Configuration Register (EPCR)

11. Document ID: US 5513376 A

L1: Entry 11 of 24
File: USPT
Apr 30, 1996

US-PAT-NO: 5513376
DOCUMENT-IDENTIFIER: US 5513376 A

TITLE: Method of operating an extension FIFO in another device when it is full by periodically
re-initiating a write operation until data can be transferred

DATE-ISSUED: April 30, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lohmeyer; Michael G.	San Jose	CA	N/A	N/A

US-CL-CURRENT: 710/53; 365/220, 365/221, 710/2, 710/34, 710/52

AB: A configurable network interface controller provides a multi-chip FIFO extension protocol. Utilizing this protocol, FIFOs that are physically separated (e.g., in separate chips) can be made to operate as though they are a single FIFO.

L1: Entry 11 of 24
File: USPT
Apr 30, 1996

DOCUMENT-IDENTIFIER: US 5513376 A
TITLE: Method of operating an extension FIFO in another device when it is full by periodically
re-initiating a write operation until data can be transferred

DRPR:
FIG. 38 is a table that describes the EEPROM Configuration Register (EPCR) of the SCENIC device.

DEPC:
EEPROM Configuration Register (EPCR) (A<7:0>-OAh)

12. Document ID: US 5495593 A

L1: Entry 12 of 24
File: USPT
Feb 27, 1996

US-PAT-NO: 5495593
DOCUMENT-IDENTIFIER: US 5495593 A

TITLE: Microcontroller device having remotely programmable EPROM and method for programming

DATE-ISSUED: February 27, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Elmer; Thomas I.	Santa Clara	CA	N/A	N/A
Nguyen; Tuan T.	Milpitas	CA	N/A	N/A
Lin; Rung-Pan	San Jose	CA	N/A	N/A

US-CL-CURRENT: 711/103; 711/147

AB: A microcontroller device on a single integrated circuit including a central processing unit, an associated data bus and an electrically-programmable nonvolatile memory is disclosed. The nonvolatile memory contains the applications program and may be remotely programmed by way of a communication port, such as a universal asynchronous, receiver/transmitter (UART) device, utilizing a separate host computer. A second nonvolatile memory is provided which contains a control program which is executed by the central processing unit for carrying out the programming of the electrically-programmable nonvolatile memory utilizing data and address information received from the host computer over the communications port.

L1: Entry 12 of 24
File: USPT
Feb 27, 1996

DOCUMENT-IDENTIFIER: US 5495593 A
TITLE: Microcontroller device having remotely programmable EPROM and method for programming

DEPR:
The subject microcontroller device includes a main bus 72 used to transport addresses and data, which is coupled to, among other devices, core 14, UART 22, UART programming control ROM 20, RAM 13, timer/counter 80, EPROM Data Register (EPDR) 74, Memory Address Register (MAR) 68, EPROM Address Register (EPAR) 66, EPROM Control Register (EPCR) 64, and the data out port of EPROM 18. The data in port of EPROM 18 is connected to the output of EPDR 74 via EPROM data in bus 76.

DEPR:
The EPROM program control register (EPCR) 64 is used to control the programming of EPROM 18. The outputs of register EPCR 64 include signal NPGM on line 82, which is a programming strobe, connected to the NPGM input of EPROM 18. As will be explained later in greater detail, an NPGM strobe is produced when an address of EPROM 18 is to be programmed with data. The outputs of register EPCR 64 also include signal ME on line 81 which controls address multiplexer 70, as explained previously.

DEPR:
UART Programming Control ROM 20 contains 256 bytes and functions as

a shadow memory, which means the
memory cannot be accessed during the normal run mode, but only in the UART program mode and the run
time programming mode. In the UART programming mode, the 256 bytes of UART Programming Control ROM
20 are assigned addresses 0300 (hex) through 03FF (hex) of the processing unit 15 address space.
Registers EPDR 74, EPAR 66, and EPCR 64 are also mapped in the shadow memory, meaning they cannot be
accessed in normal run mode, but only in UART and run time programming modes. EPDR 74 is mapped at
address 00FA (hex) in shadow memory. EPAR 66 is mapped at address 00FC (hex) in shadow memory. EPCR
64 is mapped at address 00FE (hex) in shadow memory. Thus, the "shadow memory" addresses are part of
the processor address space in the UART programming mode and in the run time programming mode but
are removed from the address space in the normal run mode.

DEPR:
The second type of command is the Prescaler Information Command which controls the duration of the
internal program pulse NPGM on line 82 produced by register EPCR 64 (FIG. 4). The Prescaler
Information Command is a single byte command which includes four bits which identify the command
type and four bits which select a prescaler value for microcontroller 12. The prescaler value can
vary from decimal 16 (2.sup.4) to 131,072 (2.sup.17) and is selected according to the prescaler
command as indicated by Table 1, below.

DEPR:
As previously noted, EPROM 18 is preferably programmed utilizing an interactive programming
sequence. Details of the interactive programming sequence are shown in the FIG. 5 flow chart.
Initially, a software counter is set to a value of 20, as represented by blocks 90 and 92. Next, a
first attempt to program EPROM 18 with one byte of received data (block 94) is made by generating
one program pulse NPGM (FIG. 4). The EPROM Control Register (EPCR) 64 (FIG. 4) will be caused to
produce the program pulse NPGM on line 82 by software executed from control ROM 20. The duration of
this pulse will be determined by the prescaler value P obtained from a previously received Prescaler
Information Command (Table 1). The interaction of software executing from control ROM 20 with
timer/counter 80 (FIG. 4) will determine the duration of the NPGM pulse in accordance with the value
P and equation (1). The data to be programmed are held in EPDR 74. (FIG. 4).

DEPR:
Run time programming is accomplished by first increasing voltage Vpp on element 32 (FIG. 2) to
+12.75 volts. Accordingly, a system design must be implemented to automatically increase Vpp in the
event run time programming is to be carried out. Voltage sensor 34 causes the UART Program Mode
(UPM) signal on line 62 to be produced thereby enabling the data register EPCR 74 (FIG. 4), control
register EPDR 64, control ROM 20 and address register EPAR 66. Core 14 may then cause certain
sections of software stored in control ROM 20 to program predetermined critical data to
predetermined EPROM 18 addresses for later evaluation.

US-PAT-NO: 4566124
DOCUMENT-IDENTIFIER: US 4566124 A

TITLE: Pattern reading system

DATE-ISSUED: January 21, 1986

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Yamamoto; Kazuhiko	Ushiku	N/A	N/A	JPX
Saito; Taiichi	Ibaraki	N/A	N/A	JPX

US-CL-CURRENT: 382/185; 382/197, 382/316

AB: A pattern reading system by line segment approximation comprising the steps of
tracing the contour and simultaneously, seeking out as candidate extreme points the points at
which the inner products of coordinate point vectors and directional vectors at coordinate
points of the contour being traced are largest, and feeding out these candidate extreme points
as real extreme points when the differences between the inner products of the direction vectors
and the inner products of the candidate extreme points are greater than an allowance set in
advance.

DOCUMENT-IDENTIFIER: US 4566124 A
TITLE: Pattern reading system

DEPR:
Preparatory to starting the tracing, in the output switches (Sw.) 10 for extreme point with respect
to 16 directions, those for the directions D.sub.5 .about.D.sub.12 are turned ON and the other
switches are turned OFF. An inner product calculator (IPC) 11 is operated to calculate projections
(a.sub.h, e.sub.i) of the start point for 16 directions. The found values of (a.sub.h, e.sub.i) for
the 16 directions are transferred to relevant extreme point candidate inner product registers 12 and
start point coordinates for all the directions are set in extreme point candidate coordinate
registers (EPCR) 13.

DEPR:
When the aforementioned sum exceeds the allowance, a comparator (CM) 18 issues a signal which causes
the x- and y-coordinates of the extreme point candidate coordinate register (EPCR) 13 to be fed out
into the extreme point output register (EPOR) 19 and also causes the extreme point output switch 10
to be turned ON in the D.sub.i -direction and OFF in the D.sub.r -direction.

When the sum is less
than the allowance, the inner product (a.sub.h, e.sub.i) of the extreme candidate inner product
register 12 and the inner product (a.sub.j, e.sub.i) of the inner product register 16 are compared
with each other by a comparator (CM) 20. The contents in the D.sub.i -direction of the extreme point
candidate inner product register 12 are exchanged for those of the current point a.sub.j of the
contour when the inner product (a.sub.h, e.sub.i) is smaller than the inner product (a.sub.j,
e.sub.i). Nothing takes place when the inner product (a.sub.h, e.sub.i) is greater than or equal to
the inner product (a.sub.j, e.sub.i). With respect to the D.sub.i -direction in which the extreme
point output switch 10 is ON, the inner product (a.sub.j, e.sub.i) and the x- and y-coordinates of
the current point of the contour are forcedly set respectively in the registers 12 and 13.

14. Document ID: US 5852171 A

L1: Entry 14 of 24

File: EPAB

Dec 22, 1998

PUB-NO: US005852171A
DOCUMENT-IDENTIFIER: US 5852171 A
TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

PUBN-DATE: December 22, 1998

INVENTOR-INFORMATION:

NAME	COUNTRY
FUKUDOME, KENJI	US
ESMON, CHARLES T	US

INT-CL (IPC): C07K 14/705
EUR-CL (EPC): C07K014/705

AB: Human protein C and activated protein C were shown to bind to endothelium
specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent
fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane
glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC
superfamily. Like thrombomodulin, the receptor involved in protein C activation, the
endothelial cell protein C receptor (EPCR) function and message are both down regulated by
exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily
provides insights into the role of protein C in regulating the inflammatory response, and
determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 14 of 24

File: EPAB

Dec 22, 1998

DOCUMENT-IDENTIFIER: US 5852171 A
TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

FPAR:
Human protein C and activated protein C were shown to bind to endothelium specifically, selectively
and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent fashion. Expression cloning
revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding
protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the
receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function
and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a
member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the
inflammatory response, and determination of methods for pharmaceutical use in manipulating the
inflammatory response.

15. Document ID: US 5804392 A

L1: Entry 15 of 24

File: EPAB

Sep 8, 1998

PUB-NO: US005804392A
DOCUMENT-IDENTIFIER: US 5804392 A
TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

PUBN-DATE: September 8, 1998

INVENTOR-INFORMATION:

NAME	COUNTRY
ESMON, CHARLES T	US
STEARNS-KUROSAWA, DEBORAH J	US
KUROSAWA, SHINICHIRO	US

INT-CL (IPC): G01N 33/53; G01N 33/564; C07K 16/28

AB: CHG DATE=19990617 STATUS=O>Plasma EPCR has been isolated, characterized and shown to
block cellular protein C activation and APC anticoagulant activity. Plasma EPCR appears to be
about 43,000 daltons and circulates at approximately 100 ng/ml (98.4+/-27.8 ng/ml, n=22).
Plasma EPCR bound activated protein C with an affinity similar to that of recombinant soluble
EPCR (Kdapp approximately 30 nM), and inhibits both protein C activation on an endothelial cell
line and APC anticoagulant activity in a one-stage factor Xa clotting assay. Soluble plasma
EPCR appears to attenuate the membrane-bound EPCR augmentation of protein C activation and the
anticoagulant function of activated protein C. Soluble EPCR has also been detected in urine.
Levels of soluble EPCR can rise in inflammatory disease associated with vascular injury and
appear to be correlated with inflammation and disease states associated with abnormal

coagulation. Since EPCR expression is restricted to larger vessels and is usually negative in
cappillaries, these observations provide a mechanism for analyzing injury/stimulation of large
vessel endothelial cells.

L1: Entry 15 of 24

File: EPAB

Sep 8, 1998

DOCUMENT-IDENTIFIER: US 5804392 A
TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

FPAR:
CHG DATE=19990617 STATUS=O>Plasma EPCR has been isolated, characterized and shown to block cellular
protein C activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons
and circulates at approximately 100 ng/ml (98.4+/-27.8 ng/ml, n=22). Plasma EPCR bound activated
protein C with an affinity similar to that of recombinant soluble EPCR (Kdapp approximately 30 nM),
and inhibits both protein C activation on an endothelial cell line and APC anticoagulant activity in
a one-stage factor Xa clotting assay. Soluble plasma EPCR appears to attenuate the membrane-bound
EPCR augmentation of protein C activation and the anticoagulant function of activated protein C.
Soluble EPCR has also been detected in urine. Levels of soluble EPCR can rise in inflammatory
disease associated with vascular injury and appear to be correlated with inflammation and disease
states associated with abnormal coagulation. Since EPCR expression is restricted to larger vessels
and is usually negative in cappillaries, these observations provide a mechanism for analyzing
injury/stimulation of large vessel endothelial cells.

16. Document ID: WO 9820041 A1

L1: Entry 16 of 24

File: EPAB

May 14, 1998

PUB-NO: WO009820041A1
DOCUMENT-IDENTIFIER: WO 9820041 A1
TITLE: ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS

PUBN-DATE: May 14, 1998

INVENTOR-INFORMATION:

NAME	COUNTRY
ESMON, CHARLES T	N/A
GU, JIAN-MING	N/A

INT-CL (IPC): C07K 14/705
EUR-CL (EPC): C07K014/705

AB: CHG DATE=19990617 STATUS=O>The promoter of the EPCR gene has been isolated from both
murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been
demonstrated to include a region which results in selective expression in endothelial cells,
between -1 and -220 based on the positions relative to the ATG encoding the first amino acid of
the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which
selectively results in expresion in large vessel endothelial cells, as opposed to all
endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of SEQ. ID No. 1).
A thrombin responsive element has been identified in the EPCR promoter, from -337 to -345 in
the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides
2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A
serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061
of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter can be used in
combination with genes encoding other proteins, as well as shorter oligonucleotides, to
increase expression by exposure to thrombin or serum or to effect selective expression in
endothelial cells generally or preferentially in endothelial cells of the large blood vessels.
The gene control elements include elements responsive to environmental stiumuli (either
thrombin or serum); and information to determine distribution of the desired protein expression
(large vessels). Therapeutic strategies include the use of the minimal promoter (-220 to -1)
for expression in all endothelial cells, for example, for any kind of gene therapy where
systemic distribution is desired; the use of a promoter including an environmental stimuli
response element(s), for use in delivery of agents whose expression should be increased during
times of increased thrombin/platelet activation or during regional trauma; the use of the
minimal promoter including an environmental stimuli response element and the element directing
expression to large vessel endothelium, where a response to regional trauma is desirable but
only in large vessel endothelium, and the use of the minimal promoter and element directing
expression to large vessel endothelium, where expresion is specifically targeted to large
vessel endothelium but is not increased in response to any particular stimuli.

L1: Entry 16 of 24

File: EPAB

May 14, 1998

DOCUMENT-IDENTIFIER: WO 9820041 A1
TITLE: ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS

FPAR:
CHG DATE=19990617 STATUS=O>The promoter of the EPCR gene has been isolated from both murine (SEQ. ID
No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been demonstrated to include a
region which results in selective expression in endothelial cells, between -1 and -220 based on the
positions relative to the ATG encoding the first amino acid of the murine EPCR protein (nucleotides
3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expresion in large vessel
endothelial cells, as opposed to all endothelial cells, located between -700 and -1080 (nucleotides
2270 to 2840 of SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR

promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin or serum or to effect selective expression in endothelial cells generally or preferentially in endothelial cells of the large blood vessels. The gene control elements include elements responsive to environmental stiumuli (either thrombin or serum); and information to determine distribution of the desired protein expression (large vessels). Therapeutic strategies include the use of the minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of gene therapy where systemic distribution is desired; the use of a promoter including an environmental stimuli response element(s), for use in delivery of agents whose expression should be increased during times of increased thrombin/platelet activation or during regional trauma; the use of the minimal promoter including an environmental stimuli response element and the element directing expression to large vessel endothelium, where a response to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal promoter and element directing expression to large vessel endothelium, where expresion is specifically targeted to large vessel endothelium but is not increased in response to any particular stimuli.

17. Document ID: US 5695993 A

L1: Entry 17 of 24 File: EPAB Dec 9, 1997

PUB-NO: US005695993A
DOCUMENT-IDENTIFIER: US 5695993 A
TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

PUBN-DATE: December 9, 1997

INVENTOR-INFORMATION:

NAME	COUNTRY
FUKUDOME, KENJI	US
ESMON, CHARLES T	US

INT-CL (IPC): C12N 5/16; C07H 21/04
EUR-CL (EPC): C07K014/705

AB: CHG DATE=19990617 STATUS=O>Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 17 of 24 File: EPAB Dec 9, 1997

DOCUMENT-IDENTIFIER: US 5695993 A
TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

FPAR:
CHG DATE=19990617 STATUS=O>Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

18. Document ID: WO 9605303 A1

L1: Entry 18 of 24 File: EPAB Feb 22, 1996

PUB-NO: WO009605303A1
DOCUMENT-IDENTIFIER: WO 9605303 A1
TITLE: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

PUBN-DATE: February 22, 1996

INVENTOR-INFORMATION:

NAME	COUNTRY
FUKUDOME, KENJI	N/A
ESMON, CHARLES T	N/A

INT-CL (IPC): C12N 15/12; C07K 14/705; A61K 39/395; C12N 15/11; A61K 38/17; C07K 16/28; G01N 33/68
EUR-CL (EPC): C07K014/705

AB: Human protein C and activated protein C were shown to bind to endothelium

specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca<2+> dependent
fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane
glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC
superfamily. Like thrombomodulin, the receptor involved in protein C activation, the
endothelial cell protein C receptor (EPCR) function and message are both down regulated by
exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily
provides insights into the role of protein C in regulating the inflammatory response, and
determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 18 of 24

File: EPAB

Feb 22, 1996

DOCUMENT-IDENTIFIER: WO 9605303 A1
TITLE: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

FPAR:
Human protein C and activated protein C were shown to bind to endothelium specifically, selectively
and saturably (Kd=30 nM, 7000 sites per cell) in a Ca<2+> dependent fashion. Expression cloning
revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding
protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the
receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function
and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a
member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the
inflammatory response, and determination of methods for pharmaceutical use in manipulating the
inflammatory response.

19. Document ID: US 6037450 A

L1: Entry 19 of 24

File: DWPI

Mar 14, 2000

DERWENT-ACC-NO: 2000-246200
DERWENT-WEEK: 200023

TITLE: Modified endothelial protein C receptor, useful as diagnostic marker of e.g. inflammation or
autoimmune diseases, is a soluble or alternatively spliced form of the receptor

INVENTOR: ESMON, C T; KUROSAWA, S ; STEARNS-KUROSAWA, D J

PRIORITY-DATA: 1997US-0884203 (June 27, 1997), 1998US-0082021 (May 20, 1998)

PATENT-FAMILY:
PUB-NO

PUB-DATE
LANGUAGE
PAGES
MAIN-IPC

US 6037450 A
March 14, 2000
N/A
024
C07K014/705

INT-CL (IPC): C07K 14/705

AB: NOVELTY - Modified endothelial protein C receptor (I) comprises amino acids (aa)
16-238 of a 238 aa sequence (S2) given in the specification, and has the C-terminal Cys
replaced by some other aa, or is not palmitoylated., DETAILED DESCRIPTION - INDEPENDENT CLAIMS
are also included for the following:, (1) isolated, natural alternatively spliced form (Ia) of
(I), comprising (S2) with a protein insertion after Gly 201; and, (2) isolated, natural form
(Ib) of (I) comprising residues 16-201 of (S2) that has been cleaved, in plasma, at a protease
site before the transmembrane domain., ACTIVITY - None given., MECHANISM OF ACTION - (I) blocks
activation of cellular protein C and anticoagulant activity of activated protein C., USE - (I)
blocks activation of cellular protein C and anticoagulant activity of activated protein C.
Measurement of (I), in standard immunoassays, is used to diagnose disorders involving
coagulation or inflammation, or autoimmune diseases (e.g. lupus erythematosus; sepsis;
diabetes; pre-eclampsia; restenosis and many others) also for monitoring treatment. (I) is a
marker of endothelial cells so its presence may also indicate damage to large blood vessels,
e.g. drug-induced damage. The antibodies used for immunoassay may also be used to characterize
and isolate receptor proteins and to modulate receptor protein activity, particularly to
inhibit binding to ligands.

L1: Entry 19 of 24

File: DWPI

Mar 14, 2000

DERWENT-ACC-NO: 2000-246200
DERWENT-WEEK: 200023

TITLE: Modified endothelial protein C receptor, useful as diagnostic marker of e.g. inflammation or
autoimmune diseases, is a soluble or alternatively spliced form of the receptor

ABTX:
NOVELTY - Modified endothelial protein C receptor (I) comprises amino acids (aa) 16-238 of a 238 aa
sequence (S2) given in the specification, and has the C-terminal Cys replaced by some other aa, or
is not palmitoylated.

20. Document ID: EP 1107790 A1, WO 200010609 A1, AU 9959013 A

L1: Entry 20 of 24

File: DWPI

Jun 20, 2001

DERWENT-ACC-NO: 2000-224557
DERWENT-WEEK: 200135
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TITLE: Specific delivery to nuclei of large vessel endothelial cells, useful for treatment or
diagnosis of cardiovascular disease, by targeting the endothelial cell protein C receptor

INVENTOR: ESMON, C T; XU, J

PRIORITY-DATA: 1998US-0139425 (August 25, 1998)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
EP 1107790 A1	June 20, 2001	E	000	A61K045/06
WO 200010609 A1	March 2, 2000	E	023	A61K045/06
AU 9959013 A	March 14, 2000	N/A	000	A61K045/06

INT-CL (IPC): A61K 45/06

AB: NOVELTY - A method of selectively delivering molecules (I) to the nucleus of
endothelial cells of large vessels by administering a conjugate (C) of (I) with an agent (II)
that binds selectively to endothelial protein C receptor (EPCR)., DETAILED DESCRIPTION - An
INDEPENDENT CLAIM is also included for a conjugate of (I) which is not a diagnostic label, with
either protein C, optionally activated, or an antibody reactive with EPCR, or fragments of it
which bind EPCR., ACTIVITY - Antithrombotic; fibrinolytic; antiinflammatory; anticoagulant;
vasoconstrictor., MECHANISM OF ACTION - EPCR translocates from the plasma membrane to the
nucleus, and transports molecules that bind to it., USE - (C) are used to deliver therapeutic
agents, e.g. nucleic acids, proteins, expression inhibitors, anti-inflammatories,
anticoagulants, growth hormones etc., or diagnostic agents such as radiolabels, fluorescent
labels and enzymatic labels, particularly in the cases of cardiovascular disease. Typical
applications include preventing thrombosis, increasing fibrinolytic activity, or inhibiting
leukocyte adhesion., ADVANTAGE - Since EPCR is present mainly on endothelial cells of large
vessels, rather than similar cells in capillaries or other small vessels, especially in
arteries, using it as target provides selective delivery of (I) with reduced risks of systemic
complications. Serum stimulates nuclear translocation of EPCR, so delivery is enhanced during
inflammatory or coagulative processes, often present in the treated subjects.

L1: Entry 20 of 24

File: DWPI

Jun 20, 2001

DERWENT-ACC-NO: 2000-224557
DERWENT-WEEK: 200135
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TITLE: Specific delivery to nuclei of large vessel endothelial cells, useful for treatment or
diagnosis of cardiovascular disease, by targeting the endothelial cell protein C receptor

ABTX:
NOVELTY - A method of selectively delivering molecules (I) to the nucleus of endothelial cells of
large vessels by administering a conjugate (C) of (I) with an agent (II) that binds selectively to
endothelial protein C receptor (EPCR).

ABTX:
DETAILED DESCRIPTION - An INDEPENDENT CLAIM is also included for a conjugate of (I) which is not a
diagnostic label, with either protein C, optionally activated, or an antibody reactive with EPCR, or
fragments of it which bind EPCR.

ABTX:
MECHANISM OF ACTION - EPCR translocates from the plasma membrane to the nucleus, and transports
molecules that bind to it.

ABTX:
ADVANTAGE - Since EPCR is present mainly on endothelial cells of large vessels, rather than similar
cells in capillaries or other small vessels, especially in arteries, using it as target provides
selective delivery of (I) with reduced risks of systemic complications. Serum stimulates nuclear
translocation of EPCR, so delivery is enhanced during inflammatory or coagulative processes, often
present in the treated subjects.

21. Document ID: EP 1033031 A1, DE 19751465 A1, WO 9927706 A1, DE 19751465 C2

L1: Entry 21 of 24

File: DWPI

Sep 6, 2000

DERWENT-ACC-NO: 1999-328210
DERWENT-WEEK: 200044
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TITLE: Sensitivity values determination unit for copying images taken by digital camera

INVENTOR: FINDEIS, G; FUERSICH, M ; KEUPP, W

PRIORITY-DATA: 1997DE-1051465 (November 20, 1997)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
EP 1033031 A1	September 6, 2000			

G
000
H04N001/60
DE 19751465 A1
May 27, 1999
N/A
005
H04N001/40
WO 9927706 A1
June 3, 1999
G
000
H04N001/60
DE 19751465 C2
September 2, 1999
N/A
000
H04N001/40

INT-CL (IPC): H04N 1/32; H04N 1/40; H04N 1/60

AB: NOVELTY - The unit comprises a recognition module (EP,CR) of the type of digital camera (KT1...N) which has taken the image. A control (CR) determines the sensitivity values in dependence on the recognized type. There is a memory (SP2) for several copying data sets (GD1...N), containing sensitivity values for image copying from different camera types.,
DETAILED DESCRIPTION - An INDEPENDENT CLAIM is included for a determination method., USE - For fotoprinter, minilab or computer controlled printer., ADVANTAGE - Precise and naturally true reproduction of images taken by digital camera., DESCRIPTION OF DRAWING(S) - The figure presents example of the unit., recognition module EP,CR, camera type KT, control CR, copying data sets. GD

L1: Entry 21 of 24
File: DWPI
Sep 6, 2000

DERWENT-ACC-NO: 1999-328210
DERWENT-WEEK: 200044
COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Sensitivity values determination unit for copying images taken by digital camera

ABTX:
NOVELTY - The unit comprises a recognition module (EP,CR) of the type of digital camera (KT1...N) which has taken the image. A control (CR) determines the sensitivity values in dependence on the recognized type. There is a memory (SP2) for several copying data sets (GD1...N), containing sensitivity values for image copying from different camera types.

ABTX:
recognition module EP,CR

22. Document ID: JP 2001508876 W, US 5804392 A, WO 9900673 A1, AU 9882694 A, EP 991946 A1

L1: Entry 22 of 24
File: DWPI
Jul 3, 2001

DERWENT-ACC-NO: 1998-505645
DERWENT-WEEK: 200142
COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Immuno-based detection of protein C receptor - useful in the diagnosis of inflammatory and coagulation states and disorders associated with damage to endothelium and large blood vessel disease

INVENTOR: ESMON, C T; KUROSAWA, S ; STEARNS-KUROSAWA, D J

PRIORITY-DATA: 1997US-0884203 (June 27, 1997)

PATENT-FAMILY:
PUB-NO
PUB-DATE
LANGUAGE
PAGES
MAIN-IPC
JP 2001508876 W
July 3, 2001
N/A
058
G01N033/53
US 5804392 A
September 8, 1998
N/A
023
G01N033/53
WO 9900673 A1
January 7, 1999
E
000
G01N033/68
AU 9882694 A
January 19, 1999
N/A
000
G01N033/68
EP 991946 A1
April 12, 2000
E
000
G01N033/68

INT-CL (IPC): C07K 14/705; C07K 16/28; G01N 33/53; G01N 33/564; G01N 33/68

AB: An assay for soluble endothelial protein C receptor comprises containing a sample from a patient to be tested and measuring the amount of soluble endothelial protein C receptor., Also claimed is a kit for detection and measurement of endothelial protein C receptor comprising:, (a) an antibody immunoreactive with endothelial protein C receptor;, (b) reagents to detect a reaction between the Ab and endothelial protein C receptor in a patient sample; and, (c) standards to correlate the amount of reaction to normal and abnormal levels of endothelial protein C receptor., USE - The assay is used for the diagnosis of coagulation and inflammatory states and disorders, damage to endothelium, and large blood vessel disease, e.g. autoimmune diseases, transplantation, sepsis, shock, pre-eclampsia, diabetes, vascular disease (especially cardiopulmonary bypass, unstable angina, restenosis and angioplasty), kidney disease and liver disease (claimed). Protein C is involved in the regulation of a host response to inflammation. The protein is one of the last components to be activated in the coagulation system, and is thought to control coagulation and inflammation. Activation of the receptor through a pathway involving thrombin, activates protein C. The protein C

pathway is apparently
only involved in large blood vessels, not capillaries, and so is activated with for major
vascular conditions, and the increased presence of the receptor in the conditions stated makes
it ideal as a diagnostic component.

L1: Entry 22 of 24
File: DWPI
Jul 3, 2001

DERWENT-ACC-NO: 1998-505645
DERWENT-WEEK: 200142
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TITLE: Immuno-based detection of protein C receptor - useful in the diagnosis of inflammatory and
coagulation states and disorders associated with damage to endothelium and large blood vessel
disease

ABTX:
An assay for soluble endothelial protein C receptor comprises containing a sample from a patient to
be tested and measuring the amount of soluble endothelial protein C receptor.

ABTX:
Also claimed is a kit for detection and measurement of endothelial protein C receptor comprising:

ABTX:
(a) an antibody immunoreactive with endothelial protein C receptor;

ABTX:
(b) reagents to detect a reaction between the Ab and endothelial protein C receptor in a patient
sample; and

ABTX:
(c) standards to correlate the amount of reaction to normal and abnormal levels of endothelial
protein C receptor.

23. Document ID: US 6200751 B1, WO 9820041 A1, AU 9854317 A, EP 937104 A1, AU 719629 B, JP 2001503273 W

L1: Entry 23 of 24
File: DWPI
Mar 13, 2001

DERWENT-ACC-NO: 1998-286871
DERWENT-WEEK: 200120
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TITLE: Regulatory elements from the endothelial protein C receptor promoter - useful to direct
expression of genes or nucleotide molecules e.g. to endothelial cells or only large vessel
endothelial cells in gene therapy

INVENTOR: ESMON, C T; GU, J

PRIORITY-DATA: 1997US-0054533 (August 4, 1997), 1996US-0030718 (November 8, 1996), 1997US-0965729
(November 7, 1997)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 6200751 B1	March 13, 2001	N/A	000	C12Q001/68
WO 9820041 A1	May 14, 1998	E	069	C07K014/705
AU 9854317 A	May 29, 1998	N/A	000	C07K014/705
EP 937104 A1	August 25, 1999	E	000	C07K014/705
AU 719629 B	May 11, 2000	N/A	000	C07K014/705
JP 2001503273 W	March 13, 2001	N/A	028	C12N015/09

INT-CL (IPC): C07H 21/04; C07K 14/705; C12N 15/09; C12N 15/12; C12P 21/02; C12Q 1/68

AB: Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR)
promoter which directs expression selectively to endothelial cells are new. Also claimed are
constructs for heterologous gene expression comprising (I), USE - The regulatory elements are
useful to control expression of a gene/biologically active nucleotide molecule (claimed), by
expressing these under control of one of the elements (optionally with the thrombin response
element) (claimed). Expression of the gene/nucleotide molecule is selectively in large vessel
endothelial cells and/or as a result of environmental stimuli (either thrombin or serum) can be
achieved by inclusion of the appropriate regulatory element(s). Atherosclerosis and most other
vascular diseases primarily occur in large vessels, and for gene therapy for such diseases it
is desirable to target endothelial cells, the primary defence mechanism against cellular
infiltration and thrombosis. The constructs are therefore particularly useful in gene therapy,
especially when the gene encodes a protein, or the nucleotide molecules are antisense, triplex
forming, ribozymes or guide sequences for RNAase P (claimed) which are used to mutate or stop
transcription of a particular gene. Such genes/nucleotide molecules may be expressed in vivo in
patients or in cell culture (claimed). For example, endothelial response elements may be used
for any gene therapy where systemic distribution is required, whilst large vessel endothelial
cell response elements are useful for expression of thrombomodulin in large vessel endothelium
to decrease clot propensity at atheromas or in autoimmune diseases; the environmental stimuli
response element(s) are useful e.g. to deliver agents whose expression

should be increased
during increased thrombin/platelet activation or regional trauma. The regulatory elements are
also useful as hybridisation probes, in increasing expression of recombinant proteins by
exposure of the encoding construct to thrombin and in drug screening and design (not claimed).,
Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR) promoter which
directs expression selectively to endothelial cells are new. Also claimed are constructs for
heterologous gene expression comprising (I), USE - The regulatory elements are useful to
control expression of a gene/biologically active nucleotide molecule (claimed), by expressing
these under control of one of the elements (optionally with the thrombin response element)
(claimed). Expression of the gene/nucleotide molecule is selectively in large vessel
endothelial cells and/or as a result of environmental stimuli (either thrombin or serum) can be
achieved by inclusion of the appropriate regulatory element(s). Atherosclerosis and most other
vascular diseases primarily occur in large vessels, and for gene therapy for such diseases it
is desirable to target endothelial cells, the primary defence mechanism against cellular
infiltration and thrombosis. The constructs are therefore particularly useful in gene therapy,
especially when the gene encodes a protein, or the nucleotide molecules are antisense, triplex
forming, ribozymes or guide sequences for RNAase P (claimed) which are used to mutate or stop
transcription of a particular gene. Such genes/nucleotide molecules may be expressed in vivo in
patients or in cell culture (claimed). For example, endothelial response elements may be used
for any gene therapy where systemic distribution is required, whilst large vessel endothelial
cell response elements are useful for expression of thrombomodulin in large vessel endothelium
to decrease clot propensity at atheromas or in autoimmune diseases; the environmental stimuli
response element(s) are useful e.g. to deliver agents whose expression should be increased
during increased thrombin/platelet activation or regional trauma. The regulatory elements are
also useful as hybridisation probes, in increasing expression of recombinant proteins by
exposure of the encoding construct to thrombin and in drug screening and design (not claimed).

L1: Entry 23 of 24 File: DWPI Mar 13, 2001

DERWENT-ACC-NO: 1998-286871
DERWENT-WEEK: 200120
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TITLE: Regulatory elements from the endothelial protein C receptor promoter - useful to direct
expression of genes or nucleotide molecules e.g. to endothelial cells or only large vessel
endothelial cells in gene therapy

ABTX:
Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR) promoter which
directs expression selectively to endothelial cells are new. Also claimed are constructs for
heterologous gene expression comprising (I)

ABEQ:
Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR) promoter which
directs expression selectively to endothelial cells are new. Also claimed are constructs for
heterologous gene expression comprising (I)

24. Document ID: AU 707349 B, WO 9605303 A1, AU 9532723 A, EP 777731 A1, US 5695993 A, US 5852171 A

L1: Entry 24 of 24 File: DWPI Jul 8, 1999

DERWENT-ACC-NO: 1996-139699
DERWENT-WEEK: 199938
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TITLE: Isolated endothelial cell protein C/activated protein C receptor - used to inhibit
inflammatory responses, screen for cpds. which alter receptor binding and, by blocking receptor
binding, enhance inflammatory response

INVENTOR: ESMON, C T; FUKUDOME, K

PRIORITY-DATA: 1994US-0289699 (August 12, 1994), 1997US-0878283 (June 18, 1997)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
AU 707349 B	July 8, 1999	N/A	000	C12N015/12
WO 9605303 A1	February 22, 1996	E	058	C12N015/12
AU 9532723 A	March 7, 1996	N/A	000	C12N015/12
EP 777731 A1	June 11, 1997	E	000	C12N015/12
US 5695993 A	December 9, 1997	N/A	028	C12N005/16
US 5852171 A	December 22, 1998	N/A	000	C07K014/705

INT-CL (IPC): A61K 38/17; A61K 39/395; C07H 21/04; C07K 14/705; C07K 16/28; C12N 5/16; C12N 15/11; C12N 15/12; G01N 33/68

AB: Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also

claimed are: (1) a nucleotide sequence encoding EPCR; and (2) an antibody or fragment
specifically immunoreactive with a unique epitope of EPCR., USE - EPCR and substances which
up-regulate its expression are useful to inhibit inflammatory responses (claimed). This
inhibition is useful in the treatment of, e.g. Gram-negative sepsis, stroke, thrombosis, septic
shock, ARDS and pulmonary emboli. EPCR is also useful to screen for cpds. which alter its
binding to (activated) protein C (claimed). Localising EPCR to surfaces in contact with blood
will render the surfaces anticoagulant as EPCR binds and concentrates the anticoagulant
activated protein C at the surface. Its function can also be enhanced by overexpressing EPCR in
endothelium that could be used to coat vascular grafts in patients with vascular disease, or in
stents in cardiac patients. Using blocking cpds. to prevent (activated) protein C binding to
EPCR it is possible to enhance an inflammatory response and so treat solid tumours., Isolated
endothelial cell protein C/activated protein C receptor (EPCR) is new. Also claimed are: (1) a
nucleotide sequence encoding EPCR; and (2) an antibody or fragment specifically immunoreactive
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(claimed). Localising EPCR to surfaces in contact with blood will render the surfaces
anticoagulant as EPCR binds and concentrates the anticoagulant activated protein C at the
surface. Its function can also be enhanced by overexpressing EPCR in endothelium that could be
used to coat vascular grafts in patients with vascular disease, or in stents in cardiac patients.
Using blocking cpds. to prevent (activated) protein C binding to EPCR it is possible to enhance an
inflammatory response and so treat solid tumours.

L1: Entry 24 of 24

File: DWPI

Jul 8, 1999

DERWENT-ACC-NO: 1996-139699
DERWENT-WEEK: 199938

TITLE: Isolated endothelial cell protein C/activated protein C receptor - used to inhibit
inflammatory responses, screen for cpds. which alter receptor binding and, by blocking receptor
binding, enhance inflammatory response

ABTX:
Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also claimed are:
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